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Instituto Superior de Agronomia
Universidade Técnica de Lisboa

# Cellular and substrate-specific interactions characterising some of the esca pathogens of grapevine

**João Daniel Condeço Oliveira**

Dissertação para obtenção do Grau de Mestre em
**Biologia Funcional**

Orientador: Doutor Ricardo Manuel de Seixas Boavida Ferreira
Co-orientador: Doutor Damiano Vesentini

**Júri:**
Presidente: - Doutora Sara Barros Queiroz Amâncio, Professora Associada do Instituto Superior de Agronomia da Universidade Técnica de Lisboa.

Vogais: - Doutor Ricardo Manuel de Seixas Boavida Ferreira, Professor Catedrático do Instituto Superior de Agronomia da Universidade Técnica de Lisboa,

- Doutora Maria Helena Mendes da Costa Ferreira Correia de Oliveira, Professora Associada do Instituto Superior de Agronomia da Universidade Técnica de Lisboa.

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"Ora, assim como a Natureza dá ao cultivador a terra e o grão, ao microbiologista o agar-agar e o esporo, de igual modo fornece ao alquimista o terreno metálico próprio e a semente que convêm."

*Fulcannelli* in "As Mansões Filosofais"

# ABSTRACT

Esca is a grapevine trunk disease, caused by one association of fungi. *Phaeomoniella chlamydospora* is presumably the first fungus to infect grapevine. Other fungus that belongs to the complex is *Fomitiporia mediterranea*. In this study *Trametes versicolor* was used as a control because it is used as model organism in other studies. Whoever it's capable of degraded wood. Exist the possibility of the wood of *Ficus carica* have properties anti fungal, however little is knowh about this matter. The wood of *Prunus* was also contemplated on this study. The tree fungis where subjected to anaerobic conditions, and do not present any grow. The fungis produces $H_2O_2$ and ROS when they interact between them. The interaction of *Phaeomoniella chlamydospora* and *Trametes versicolor* produces one big mark of interaction visible even whiteout reagents. The phenotype of aerial hyphae is altered. Fungi have demonstrated capacity to degrade wood of the different species. The degradation was observed on the microscope using different methods. The wood was also used to produce different means of agar using dust and extract of wood. For every case where obtain different rates of growth and degradation. The tree fungi have the ability to degrade wood. The peripheral growth of the hyphae was observed in different media, the phenotype have no alterations, but the length of the hyphae have alterations.

Key-words: Esca; Wood; Degradation, Fungus; Growth; Interactions

# RESUMO

A esca é uma doença do lenho da videira causada por uma associação de fungos. Pensa-se que o primeiro fungo a invadir seja a *Phaeomoniella chlamydospora*. Um outro fungo associado a esca é a *Fomitiporia mediterranea*. *Trametes vericolor* é um fungo que degrada madeira não associado a esca. Existe a possibilidade da madeira de *Ficus carica* ter propriedades antifungicas, no entanto muito pouco se sabe sobre este assunto. O lenho de *Prunus* serviu de madeira controlo. Os fungos foram sujeitos a condições anaeróbicas e não apresentaram crescimento. Os fungos produzem $H_2O_2$ e ROS quando interagem entre si. A interacção entre *Phaeomoniella chlamydospora* e *Trametes versicolor* produz uma grande mancha de interacção, o fenótipo deste ultimo chega a ser alterado, observando-se menos hífas aéreas. Os fungos demonstraram capacidade de degradar a madeiras das diferentes espécies vegetais. A degradação das madeiras foi observada ao microscópio segundo diferentes métodos. A partir das madeiras foram criados meios de agar à base de pó de madeira e de extracto de madeira. Para cada caso foram obtidas diferentes taxas de crescimento e degradação. O crescimento periférico das hífas foi observado em diferentes meios, embora não haja alteração no fenótipo, existem diferenças no comprimento das hifas.

Palavras-chave: Esca; Madeira; Degradação; Fungos; Crescimento; Interacção

# RESUMO ALARGADO

A esca é uma doença do lenho da videira (*Vitis vinífera*), causada por uma associação de fungos. A esca apodrece a madeira da videira e também causa a sua morte, razão pela qual a madeira da vinha foi estudada. Neste trabalho foram estudados dois fungos da esca, *Phaemoniella chlamydospora* e *Fomitiporia mediterranea*. Pensa-se que *P. chlamydospora* seja o primeiro fungo a invadir o lenho da videira. Posteriormente a madeira é invadida por outros fungos, incluindo *F. mediterranea*. Como fungo controlo foi usado para este estudo *Trametes versicolor* um fungo que causa grandes prejuízos na construção civil ao apodrecer a madeira. Este fungo encontra-se muito bem caracterizado seno por isso utilizado em muitos estudos como organismo controlo. Na Natureza encontra-se em madeiras de árvores mortas, tendo grande importância na degradação das mesmas e na reciclagem de nutrientes. A madeira da figueira (*Ficus carica*) possui uma densidade próxima da madeira da videira e por isso foi contemplada neste estudo como madeira controlo. Complementarmente a madeira de figueira será estudada para perceber se existe nesta potencial anti-fúngico. Outra madeira controlo utilizada foi a madeira proveniente de uma árvore do género *Prunus* não foi possível identificar a espécie em concreto, no entanto a sua densidade é próxima da densidade do lenho de videira. Neste estudo observou-se que os fungos não crescem em anaerobiose, permanecendo no entanto vivos o que indica que são aeróbios facultativos. As madeiras foram sujeitas a testes de degradação por parte dos fungos. Em todos os casos ocorreu perda de massa da madeira. As madeiras degradadas foram observadas a lupa e ao microscópio segundo várias técnicas microscópicas. As observações histológicas revelaram os danos a nível das paredes celulares das madeiras e a presença das hífas dos fungos. É possível que *P. chlamydospora* tenha capacidade para degradar a madeira morta. O fungo *F. mediterranea* pode muito provavelmente infectar o lenho da videira sem o suporte de *P. chlamydospora*, poderá acontecer portanto que nalguns casos este fungo possa ser o primeiro da sequência. Também foram estudadas as interacções entre fungos, nomeadamente a producção de $H_2O_2$ e de ROS. O fungo *T. versicolor* mostrou uma alteração de fenótipo visível mesmo sem análise de produção de espécies reactivas de oxigénio. Diferentes meios foram criados com base em pó de madeira e extractos líquidos do pó madeira. Nesses meios foram observados os crescimentos dos fungos. Com os mesmos meios observaram-se a estrutura e o crescimento das hífas. Não foi detectada nenhuma relação entre o crescimento dos fungos em meios, a capacidade para degradar madeira, ou o tamanho dos segmentos de hífas.

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# LIST OF ABBREVIATIONS

$HO_2$· - hydroxyperoxyl radical

$H_2O_2$ - hydrogen peroxide

HSFs - heat-shock transcription factors

Hsr - hypersensitive response

L - length between the inoculum and the terminal zone of the fungal colony

LRR - leucine-rich repeated domain

MAMPs - microbe-associated molecular patterns

$M_i$ - initial mass

$M_f$ - final mass

N - number of measurements

NO - nitric oxide

PDA - potato dextrose agar medium

PEG - polyethylene glycol

$OH^-$ - hydroxyl radical

$O^·$ - singlet oxygen

$O_2^-$ - superoxide anion

$O_2^{2-}$ - peroxide ion

PAMPs - pathogen-associated molecular patterns

ROS - reactive oxygen species

Δt - time of growth in hours

# 1. INTRODUCTION

Until the 1990s esca was considered a disease of old grapevines. At that time, studies began to point out that the some pathogens associated to esca were also responsible for the decline of young grapevines. Laura Mugnai suggested that esca might have originated at the time of ancient Greece and Roma, and that indeed it may be even older than vine cultivation itself. The disease is so complex that it is associated with many symptoms, some of which give their name to the disease in some regions of the world (Mugnai 1999).

The symptoms of esca disease can, in some cases, be very similar to those characteristic of other grapevine diseases, although they have different origins. Esca can manifest itself in spring, but this is something rare. Normally the external symptoms occur in summer between June and September, in the Northern Hemisphere (Surico *et al.*, 2000). Further details on esca symptoms will be described in the next section.

Esca became a widespread concern in the 1990s, when arsenite was banned. Arsenite containing fungicides had been, until that time, the only known method for controlling esca. In this situation other less efficient products had to be used, which provided poor results. Esca has been detected in countries where viticulture is a well established agricultural practice and an important economic sector, such as Portugal, Italy, Spain, Germany, Greece, as well as California and the United States in general (Mugnai *et al*., 1999).

### 1.1. State-of-the-art on esca

Esca is a disease caused by one association of fungi that has been discovered at the end of the XIX century and has gained considerable interest throughout the XX century. It has been suggested that esca manifests itself in two stages. In the first stage, the plant woody tissues are sequentially colonized by a number of microorganisms, which lead to the formation of a central light-coloured soft necrosis. The first organisms to colonize are *Phaeomoniella chlamydospora* and *Phaeoacremonium* spp. In the second stage the wood is invaded by fungi from the genus *Fomitiporia* this can be fallowed by other species of fungus (Larignon & Dubos, 1997).

Two types of esca can be identified, the chronic type and the acute type. The chronic syndrome affects adult plants (more than 8-10 years of age), and is characterized by the occurrence of a white rot in the internal wood, which gradually changes to soft rot. This soft wood appears as a spongy material, which normally has a yellow or white colour delimited by a black or dark brown line (Baldacci *et al.*, 1962). This line separates the degraded from the asymptomatic wood. Sometimes the rot reaches the external surface of the wood and creates fissures. Although the rot spreads along the trunk, it does not pass the graft union,

nor does it not affect the root system. Despite this, American grapevines and Euro-American hybrids are not immune to esca, because the same symptoms may develop in ungrafted vines of either type (Mugnai *et al.*, 1999).

The leaf may develop symptoms which consist of chlorotic rounded or irregular spots between veins. These chlorotic spots grow and with time become necrotic, leaving only part of living tissue along the veins. Consequently, the affected tissue becomes yellow-brown and/or red-brown. In this situation leaves assume the typical “tiger-stripes” pattern (Mugnai *et al.*, 1999), which is commonly reported in association with the disease. Although the real cause of this characteristic pattern remains unknown, it is possible that this symptom is caused by reduced hydraulic conductivity in the xylem. However, it is worth of consideration that even if the xylem is partially blocked, this would not fully explain the symptoms observed in the leaves. Normally, the functional wood is not affected in terms of water transport. Furthermore, even a partial blockage could not inhibit the functionality of such a complex water translocation system such as the xylem. The change in water potential associated to cavitation may affect grape production. However, it is likely that the substances that cause the degradation of wood are translocated to the leaves where they can induce the necrosis observed in the leaf tissues (Sparapano *et al.,* 1998).

The grape symptoms include dark brown spots, which can vary to a violet or purple colour. The grapes dry and are easily attacked by fungi and bacteria. These symptoms may appear in isolated grapes or can affect all the grapes of a plant. The origin of such symptoms may be related to the occurrence of enzymes associated with the pathogen, or more probably, to the effect of toxins produced by the fungi in the wood, and then transported to leaves and berries (Sparapano *et al.*, 1998; Mugnai *et al.*, 1999). In young plants (less than 8 to 10 years old) esca is usually associated with limited wood discoloration. White rot in young vines is rare and this symptom is generally associated with older plants. The white colour is caused by physical and chemical changes in the wood, including:

1) Physical and chemical alterations caused by the introduction of air and water through wounds into the wood, as well as host reactions that induce degradation or oxidation of wood components (Baldacci *et al*., 1962).

2) Lignolytic and cellulolytic enzymes produced by the fungi (Chiarappa, 1959).

3) Induction of tylose synthesis, segregation of gums by diseased tissues, production of high-molecular weigh compounds by fungi, and necrosis induced by fungal toxins or by chemical defences of the plant (Sparapano *et al.*, 1998).

In the acute syndrome, also known as apoplexy, the vine dies within a few days. This generally takes place in summer, when the weather is very hot, especially after a rain event.

It may be that under these conditions, the concentration of toxic compounds rapidly rises within the plant, whereby the elevated level of transpiration facilitates a rapid translocation of these compounds to the leaves and berries (Bruno *et al.*, 2007).

## 1.2. Fungal attack and plant defences

### *1.2.1. Saprophytic vs. pathogenic fungal interactions*

Saprophytic fungi interact with the plant in a different way from that of pathogenic fungi. The hyphae of pathogenic fungi penetrate the cell wall of plants by means of the formation of an appressorium and a penetration peg. Consequently, the point of penetration becomes almost indistinct in the initial phases. Then the fungus degrades the cell wall, and penetrates inside the cell. At the point of penetration a clear strangulation of the hyphae occurs. At the same time, the cytoplasm moves in the direction of the hyphae. This was evidenced by the movement of mitochondria, endoplasmic reticulum and polyribosomes. However, this may be associated with other alterations in the plant metabolism (Beswettherick and Bishop, 1993).

The hypha of saprophyte fungi secretes pectinolytic enzymes and organic acids. These enzymes and acids degrade the pectin substances of middle lamella. As a result the cell loses the cohesion to the surrounding tissue, and the area becomes soft. The fungi also release cellulases that break down the cell wall, disintegrating the cell. The mycelia do not invade the cells, rather they live on substances liberated from the dead cells (Agrios, 2004).

Plants can initiate the cascade of biochemical events associated with defence mechanisms, by detecting specific pathogen-related substances known as elicitors. Elicitors can originate in the pathogenic fungus (exogenous elicitors), or in the plant itself (endogenous elicitors), which may have experienced alterations in their biochemical composition because of the pathogen. These include, for example, low molecular weight compounds deriving from plant cell wall degradation. However, endogenous elicitors are often associated with viral and bacterial interactions. The plant receptors for fungal elicitors are generally located in the plasma membrane. Some elicitors are pathogen-specific or plant-specific and are encoded by *avr* genes. Normally the presence of elicitors is associated with a hypersensitive response (hsr). As an example, botrycin and cinerein isolated from *Botrytis cinerea*, cause the formation of necrotic lesions and a typical *hsr*. These elicitors in leaves activate MAP kinases, induce the transcription of genes encoding enzymes involved in the phenylpropanoid pathway, and induce the ion influxes across the plasma membrane and production of reactive oxygen species (ROS) (Nürnberger, 1999).

Fungi have also developed mechanisms of host recognition. Obligate pathogens can only infect one species, or a selected small group of closely related species. In this way they had to develop finely tuned mechanisms of recognition which include for example

recognition of the defence mechanisms of the plant, which triggers the activation of genes necessary for pathogenicity. Other signals such as hydrophobicity, biochemical composition and hardness of the surface also play an important role in host recognition (Tyler, 2002).

Plants possess a wide array of proteins which play a crucial role in pathogen recognition. Recognition can take place via binding of pathogen-specific molecules, hence for example the leucine-rich repeated domain (LRR) of some proteins. In some cases the protein is located in the membrane, with the LRR domain oriented towards the extracellular zone. In this case, the protein recognises microbe-associated molecular patterns (MAMPs) and pathogen-associated molecular patterns (PAMPs) (Nürnberger and Kemmerling, 2006). However, in most cases, the LRR domain is located intracellularly and identifies subtle perturbations inside the cell. Examples of perturbations inside cells include the presence of microbial compounds and the presence of cytochrome *c* outside mitochondria (Leipe *et al*., 2004). In many cases LRR domains are coupled with a nucleotide binding domain. Although these domains are very similar, it is believed that they have different origins. Even in animals, LRR domains are fused with binding nucleotide domains and serve to detect pathogens (Rairdan and Moffett, 2007). The LRR domain is constituted by a peptide motif that presents the following sequence pattern: LxxLxxLxLxxNxLxGxIPxxLGx (L, leucine; N, asparagine; G, glycine; I, isoleucine; P, proline, x, any amino acid) (Kajava, 1998).

Indent plant molecules that are recognised by R proteins are known as effectors. Among the substances that are known to interact with the LRR domain are chaperones and co-chaperones (Bieri *et al*., 2004; Azevedo *et al*., 2006). It has been suggested that these molecules play a role in folding the LRR domain. Nevertheless, the regulation of these proteins remains poorly understood. It has been suggested that when an R protein detects its corresponding effector, it binds to a nuclear protein and affects the activity (positively or negatively) of transcription factors that regulate the expression of defence genes (Tameling and Takken, 2008)

A further defence mechanism in plants relies on the production of ROS. ROS are highly reactive molecules or ions which originate from oxygen. The most common ROS are singlet oxygen ($O^{\cdot}$), superoxide anion ($O_2^{-}$), hydrogen peroxide ($H_2O_2$), hydroxyl radical ($OH^{-}$), hydroxyperoxyl radical ($HO_2\cdot$) and nitric oxide (NO). ROS are highly reactive and can be used against fungi. Generally, avirulent pathogens induce ROS accumulation in plants. The first step of ROS accumulation is the transient accumulation of small amounts of ROS. The second stage is a more intense and continuous phase of accumulation (Lamb and Dixon 1997). Virulent pathogens normally cause only the first step of ROS accumulation. It is thought that this type of parasites suppress the system of plant recognition (Torres *et al*., 2006).

ROS can be produced by different plant organelles according to the type of stress being experienced. In the cell, ROS-producing organelles include chloroplasts (Kariola *et al*., 2005), peroxisomes (Kużniak and Skłodowska, 2005) and mitochondria (Amirsadeghi *et al*., 2007). ROS, especially $H_2O_2$, act like anti-microbial agents (Walters, 2003; Custers *et al*., 2004). Several *in vitro* studies, performed with different concentrations of $H_2O_2$, indicate that ROS inhibit the growth of several phytophatogenic bacteria and prevent the germination of fungal spores (Peng and Kuc, 1992; Wu *et al*., 1995). It remains unclear if ROS affect fungi and other microbes because of their toxicity and/or if they exhibit some function on the activation of defence genes in the plant (Shetty *et al*., 2008). ROS are also toxic for plants and high levels can cause damage and cell death. The production of ROS is therefore regulated so that in normal situations plants do not suffer oxidative stress (Levine *et al*., 1994).

ROS are involved in every signalling pathway for defence mechanisms, such as the hypersensitive response, accumulation of phytoalexins, and other genes involved in defence responses. It was been suggested that plants sense ROS by three different mechanisms: (i) unidentified receptor proteins, (ii) redox-sensitive transcription factors or heat-shock transcription factors (HSFs), and (iii) direct inhibition of phosphatase. Although ROS signalisation has been widely studied, the process remains poorly understood (Neill *et al*., 2002; Mittler *et al*., 2004).

After pathogen attempted infection, the plant responds with a hypersensitive response, with gene defence expression, or with both. This response is mediated by changes in the ion flux, protein phosphorylation and oxidative burst (Chandra *et al*., 1996; Jabs *et al*., 1997; Sasabe *et al*., 2000). The plant starts with the changes in ion fluxes, creating one influx of $Ca^{2+}$ and $H^+$ and one efflux of $K^+$ and $Cl^-$ (McDowell and Dangl, 2000). This type of flux generates an extracellular production of ROS.

### *1.2.2. Rot fungi*

There are three basic types of wood decay, white-rot, brown-rot and soft-rot (Eaton and Hale, 1993). The white-rot degrades lignin and hemicelluloses on first place, on a second phase they degrade to the cellulose. Some white-rot fungi degrade all compounds at the same time. The oxidation of lignin creates a white colour on the wood. These fungi are the only microbes known which can degrade and mineralize lignin efficiently. Of these fungi the best characterized is *Phanerochaete chrysosporium*. There are many enzymes identified for this species that have been related with the degradation of wood, such as laccase, lignin peroxidase, manganese peroxidase and cellobiose dehydrogenase (Hatakka, 1994). The majority of these fungi are basidiomycetes, and a minor part are ascomycetes (Risna and Suhirman, 2002; Urairuj *et al*., 2003).

The brown-rot fungi degrade cellulose and hemicelluloses by non-enzymatic oxidation, they also use cellulases and glucanases. Lignin suffers little degradation (Bucher *et al*., 2004).

The soft-rot fungi make a superficial decay where polysaccharides and lignin are degraded. This type of rot is common in ascomycetes, bacteria wood decay also have this type of degradation (Bucher *et al.*, 2004).

### 1.3. Wood

*1.3.1. Wood composition and enzymatic degradation*

Wood is composed of cellulose, lignin and hemicelluloses, which are the principal components. Pectin is also a wood component, which is limited to the interface between separate cells, where the cell walls of distinct cells touch. This is also known as the middle lamella. Cellulose, hemicelluloses and lignin are more widespread and are found across the plant cell walls.

Lignin is a polyphenolic compound that is responsible for the hardness of wood and is resistant to biological attack. In the secondary cell wall, lignin (with hemicelluloses) envelops the fibrils of cellulose, and protects it against degradation. This polymer is highly concentrated in the middle lamella together with pectin, and is also abundant in the cell wall, mainly in the secondary cell wall. Lignin is a compound which is very resistant to degradation, as each lignin molecule is a unique, complex molecule, requiring the availability of specialized biochemical tools able to oxidize the phenolic part of lignin. For this reason, only some microorganisms are capable of decomposing lignin. It is estimated that about 500 fungal species have been identified that can effectively use lignin as a growth substrate (Agrios, 1997).

Cellulose represents nearly 50% of the wood mass. Cellulose is a linear polymer of glucose molecules organised in a linear β-1,4 structure. It is generally organised in microfibrils, although sometimes it assumes a crystalline form. In the secondary cell wall, lignin and hemicelluloses envelop the fibrils of cellulose, and protect it against degradation (Martínez et al., 2005).

Hemicelluloses have an intermediate degree of complexity between lignin and cellulose and are constituted by residues of pentoses and hexoses. Other compounds of the wood are phenols and tannins, as well as fats, sugars and proteins. This kind of chemicals represents 5 to 20% of the dry mass of wood (Martínez et al., 2005).

The main wood components are degraded by substances like oxalic acid, mycotoxins and other organic acids, which during the initial steps of decay attack hemicelluloses (Punja, 2001), thus opening up the wood matrix and allowing the translocation and action of cellulolytic and lignolytic enzymes, such as cellulase, glucanase (cellulose), lignin

peroxidase and laccase (lignin). Whilst it is widely accepted that cellulolytic and lignolytic enzymes play a key role in wood decay, the role of these enzymes in plant wood diseases such as esca in grapevine, remains to be fully understood (Punja, 2001).

### *1.3.2 Wood analysis other techniques*

*Histology*

Microscopy studies are a very powerful tool, allowing the examination of wood structure and the discrimination of specific biochemical components through the use of *ad hoc* hystological techniques.

Among these techniques, safranin and fast green allow distinguishing the more lignified portions of wood that are coloured red, from the less lignified portions that are coloured green. Even in advanced stages of wood degradation the differences are still visible (Jensen, 1962).

Azure B is another technique that colours lignin, but also nucleic acids. The stained tissues present a blue-green coloration (Jensen, 1962).

The presence of cellulose can be determined by the technique of the zinc-chlor-iodide. However, this technique can distort the section of wood in association with phloroglucinol technique the middle lamella appears red and the secondary walls become bluish (Vaughan, 1914).

The technique of iodine-potassium iodide colours starch in dark blue and black (Jensen, 1962).

*Mass loss*

Fungi can cause loss of wood mass. During wood degradation, cellulose, lignin and others compounds are hydrolyzed and utilized by the fungi. The wood can be colonized by fungi and subjected to degradation. After a period of time, it is possible to measure the loss of mass, by comparing the initial mass and the final mass of the wood (Bucher *et al*., 2004).

## 1.4. Fungal growth under aerobic or anaerobic conditions

The oxygen status inside grapevine wood is unknown. It is not known if esca fungi can grow under anoxic or hypoxic conditions. It has been suggested that in general, fungi need oxygen for spore germination and hyphae growth and that the absence of $O_2$ inhibits germination (Yang and Lukas, 1070; Subíková and Subík, 1974). Fungi use oxygen as a final acceptor of electrons not only in the respiratory pathway, but also in some other essential biosynthetic reactions independent of oxidative phosphorylation (Heslot and Goffeau, 1970). For example, yeast does not require oxygen during fermentation, but needs $O_2$ to live (Kellerman *et al*., 1969). Whether there is any type of growth in esca fungi in the

absence or in the presence of low concentrations of $O_2$ remains unknown; yet, this factor may have some influence on the infection of grapevine.

### 1.5. Interactions among fungi and importance of ROS

In nature fungi interact with other organisms, including other fungi. Since esca is a disease caused by the association of different fungi, the interactions occurring during esca have attracted attention in the literature (Freitas *et al*., 2008). Interactions among fungi can assume three forms, mutualistic, neutralistic and competitive (Rayner and Todd, 1979). The secondary metabolites produced by fungi during interactions are very important, as they can act as inhibitors or stimulators of the mycelial growth in other fungi (Heilmann-Clausen and Boddy, 2005). In grapevine, fungi that are in competition form zone lines, or barrage zones, in which none of the fungi can overgrow (Boddy, 2000).

It has been observed that the interaction between *Botryosphaeria obtusa* and *Eutypa lata* gives origin to *O*-methylmellein and its hydroxylated forms. These compounds do not appear in pure cultures and are only produced during fungal interactions (Glauser *et al*., 2009). *O*-Methylmellein displays antifungal activity against some fungi (in this case, it is toxic to *B. obtusa*) and is also phytotoxic. However, the hydroxylated forms of this compound are only toxic to plants, and do not present antifungal activity.

During interactions of different organisms, ROS can be produced, which play a role in the recognition of invaders (Torres *et al*., 2002; Vignais, 2002). In fungi, this production of ROS has also been described (Silar, 2005). Interacting fungi produce a pattern of ROS different from that of isolated cultures. In the study of Silar (2005), many fungi were capable of self-recognition, whilst some filamentous fungi can recognize other filamentous fungi, yeasts (only in some cases), bacteria, and wood. However none of the fungi studied reacted to the presence of dead fungi, or inert material like plastic or glass (Silar, 2005). Thus, the most prominent variation in ROS production was detected in interactions between different filamentous fungi, although other compounds may also play a role during these interactions. Fungi use ROS not only to recognise other fungi, but also to initiate wood digestion and to disrupt lignin and cellulose. Enzymes cannot penetrate wood in the beginning, so digestion is started by ROS, like OH● (Hammel *et al*., 2002).

Fungal NADPH oxidases (Lalucque and Silar, 2003), peroxidases like lignin peroxidase (Hofrichter *et al*., 1998), and laccases (Thurston, 1994; Mayer and Staples, 2002) are involved in ROS activity. NADPH oxidases produce $O_2^-$, as was evidenced by *in vitro* experiments, but *in vivo* the radical most frequently generated is peroxide ion ($O_2^{2-}$) (Sagi and Fluhr, 2001). Other enzymes produce hydrogen peroxide ($H_2O_2$) for the activity of the peroxidases, like aryl alcohol oxidase or glyoxal oxidase. However, these enzymes do not participate directly in wood digestion, but only provide the ROS (Leonowicz *et al*., 2001).

Lignin peroxidase needs $H_2O_2$ to be active. This enzyme catalyzes several reactions like the oxidation of alkyl side-chains of lignin compounds, C-C cleavages in the side chains of lignin subunits, oxidation of veratryl alcohol to aldehydes and ketones, cleavage of phenylglycol structures and hydroxylation of benzylic methylene groups (Tien and Kirk, 1983). Laccases can oxidase a variety of aromatic compounds by using them as electron and proton donors (Agematu *et al*., 1993).

It has been suggested that the fungi *F. mediterranea*, *P. chlamydospora* and *Togninia minima* (three esca fungi) use laccases, peroxidases and tannases to detoxify antimicrobial substances produced by grapevine, giving competive advantage to the fungus with the best ability to detoxify them (Bruno and Sparapano, 2006).

Esca is causing considerable damage to vineyards and economical losses to wine producers. In order to control the spread of the disease, it is important to understand the mode of action of the esca fungi, as well as to understand the corresponding resistance mechanisms in grapevine.

This study focused on *Phaeomoniella chlamydospora*, which is probably the first fungus to invade grapevine (Valtaud *et al*., 2009) and *Fomitiporia mediterranea*, since the genus *Fomitiporia* has consistently been associated with the occurrence of esca (Graniti *et al*., 2000). Different *Fomitiporia* species occur according to the geographic distribution of the disease. In Portugal *F. mediterranea* is the most recurrent species. The control fungus utilized in the present work was *Trametes versicolor*, which was used as a model organism for white-rot wood decaying fungi (Leonowicz *et al*., 2001). *Vitis vinifera* was used as their wood substrate together with *Ficus carica* and *Prunus spp*. acting as controls, since all these wood species have similar densities.

# 2. MATERIALS AND METHODS

### 2.1 Fungal cultures

The fungal species used in this study were *Trametes versicolor*, *Fomitiporia mediterranea*, and *Phaeomoniella chlamydospora*. *T. versicolor* was obtained from Dr. Lina Nunes at Laboratório Nacional de Engenharia Civil, Lisbon, Portugal, whilst *F. mediterranea* and *P. chlamydospora* were isolated from infected grapevine materials. All cultures were maintained at the Laboratório de Patologia Vegetal "Veríssimo da Almeida" of Instituto Superior de Agronomia, Lisbon, Portugal. The fungal cultures were grown on autoclaved potato dextrose agar medium (PDA), at 3.9% (w/v).

### 2.2 Wood material

The samples of wood used were from *Prunus*, *Ficus carica* and *Vitis vinifera*. The wood was cut in blocks of 30x10x5 mm for tests of degradation. In other tests, wood was made into a powder using a Wiley mill equipped with a mesh 40 screen.

### 2.3 Tests on anaerobiosis

Plates containing 20 ml of PDA were inoculated with mycelial plugs removed from the margin of actively growing colonies of the fungi under study. Each fungus was inoculated in triplicate, for each condition. Inoculated plates for each fungal species were placed under sterile conditions either inside an anaerobic assay container, on the container a producer of $CO_2$ and a $O_2$ indicator were put, a pink colour on the indicator indicates the presence of oxygen, the colour white indicate the consummation of all oxygen. The control plates were maintained at ambient atmosphere as a control. All plates were incubated at both 15 and 24 ºC for up to 28 days. At the end of this period, fungal cultures kept under anaerobic conditions were exposed to ambient atmosphere and allowed to grow, at the same temperature, for up to 31 days. Radial growth rate was then calculated and four measurements were recorded for each plate at 90 degree intervals every four days.

### 2.4 Analyses of reactive oxygen species

The presence of reactive oxygen species (ROS) was analysed for each of the three fungal species under study, either isolated or during fungal-fungal interactions, according to the method of Freitas (Freitas *et al*., 2008). The following interactions were evaluated: *T. versicolor* versus *F. mediterranea*, *T. versicolor* versus *P. chlamydospora* and *F. mediterranea* versus *P. chlamydospora*. Upon the fungal mycelia coming into contact with each other, 5 ml of ROS detection reagent was placed on each plate (including the controls)

and the solution was spread with a sterile glass spreader. Plates were incubated for 30 min, after which the solution was removed and the plates incubated for further 24 h to allow for colour development. For the detection of superoxide a reagent was used containing 2.5 mM of nitrobluetetrazolium and 5 mM of *N*-morpholinopropanesulphanate-NaOH at pH 7.6.

For the detection of hydrogen peroxide, a reagent was used containing 2.5 mM of diaminobenzidine and 5 units of purpurogallin per ml in 100 mM of potassium phosphate buffer at pH 6.9.

**2.5 Tests of wood degradation**

The blocks of wood where dried at 80 ºC for 24 h, by which time constant weight was achieved. The mass of the blocks was measured and blocks were then sterilised at the Instituto de Tecnologia Nuclear by gamma radiation to 0.5 Mrad total exposure, according to EN 113 method (Leithoff, 2010). Four sterile plastic rings were placed on plates with PDA at 3.9% (w/v) for support. The plates were then inoculated with each of the test fungi. Upon the mycelium reaching the plastic supports (3 to 5 days post-inoculation), the sterilised wood blocks were placed over the rings. Plates were incubated at 24 ºC for up to 38 days. Upon harvesting the blocks, the mycelium was separated from the wood blocks by gently scraping the surface with a scalpel. The blocks were then dried for 24 h at 80 ºC. The mass of the blocks was then measured, and differences in mass were registered (Vesentini *et al.*, 2006a). The blocks were observed under a stereomicroscope for a preliminary assessment of the damage caused by the fungi.

To observe damage in individual cells a technique of dissociation of cells was used to separate individual wood cells. Small wood shavings were placed in test-tubes containing 4 ml 1:1 (v/v) acetic acid and hydrogen peroxide. The test-tubes were topped covered with tin foil, and incubated at 60 ºC during 24 h. After that time wood solids were removed with a sieve and washed with water. Isolated wood cells were collected carefully with a pair of tweezers and placed in test-tubes with ethanol 70% (v/v). The material was then mounted in water and observed under an optical microscope, according to the Jeffrey's method (Johansen, 1940).

To observe the damage occurring at tissue level, the blocks were embedded in polyethylene glycol (PEG). PEG must previously melt at 60 ºC in an incubator with ventilation. For each individual block, 20 ml of PEG where placed in plastic cups, then 80 ml of distilled water were added and the wood blocks where placed in the cups. The cups were incubated at 60 ºC until the water evaporated. At this stage, the PEG was removed, and replaced with new PEG, so as to cover the wood. The cups were returned to the incubator for an additional 2 h. After this time, the cups containing the samples were removed from the incubator and stored in a cool dry place, but not in a refrigerator. The wood was centred and

the PEG was allowed to solidify during 24 h. Wood sections (25 µm thick) were cut in a sledge microtome from both transversal and longitudinal surfaces and then observed under an optical microscope. Four different methods where used to assess the fungal-induced damage; control (natural wood), method of phloroglucinol-HCl (lignin stain), method of methylene blue (fungal hyphae stain) and method of picroaniline blue (cellulose stain).

The observation of natural wood was made with the wood samples in distilled water.

In the method of phloroglucinol-HCl, the sample was placed in the phloroglucinol acid solution for 5 min. After that time, the wood was placed on a glass cover slide and mounted in 6 M HCl (Clifford, 1974).

In the method of methylene blue, the sample was stained in methylene blue 0.1% (w/v) for 15 min. Then the sample was washed with distilled water and water-mounted (Stevens, 1924).

In the method of picroaniline blue, the stock solution must be prepared previously. A 1% (w/v) stock solution of saffranine was prepared. To prepare picroaniline blue solution, 25 ml of saturated aqueous aniline blue were added to 100 ml of saturated aqueous picric acid. The wood samples were stained for 2 min in the saffranine solution, which was prepared with 3 drops of saffranine stock solution in 10 ml of distilled water. The wood sample was then washed in distilled water and placed in a solution of picroaniline blue, which was prepared by adding 5 drops of picroaniline blue stock solution to 10 ml of distilled water. At this stage, the sample in the picro aniline blue solution was heated until the stain began to steam slightly. The wood sample was then washed in distilled water and water-mounted for observation according to the method of Cartwrigth (1929).

**2.6 Tests of fungal development on different media**

Different media containing wood-derived substrates were prepared to test the development of the fungal species under study. All tests were performed in triplicate in sterile media, and the conditions tested included:

- Tap water containing agar 1.5% (w/v);
- PDA at 3.9 % (w/v);
- Distilled water, 20 g of pulverised wood (per plate) and 1.5% (w/v) of agar;
- Wood broth containing agar 1.5% (w/v);
- Distilled water, 20 g (per plate) of pulverised wood used to create the wood broth and 1.5% (w/v) agar.

The broth wood was created incubating by one hour the pulverised wood in distilled water at the concentration of 1 $g.ml^{-1}$.

The media thus prepared were dispensed in 90 mm Petri dishes (20 ml), which were then incubated at 24 ºC. The radial growth of fungi was measured (four measurements per plate) after 96 h and 192 h. The rate of growth per day was then calculated.

To observe hyphal growth and morphology, three sterile squares of cellophane were placed on each of three plates containing the growth media described previously. Fungi were inoculated on the cellophane and were maintained at 24 ºC. The hyphae of *T. versicolor* were observed after 42 h, whereas those of *F. mediterranea* and *P. chlamydospora* were observed after 72 h under a microscope. For observation, individual cellophane squares were placed on a microscope slide, the inoculation plug was removed and the slides were covered by a coverslip. Measurements of hyphae were made using the appropriate Leica program. The length of 45 hyphae was assessed from the tip backwards to at least the second ramification. The total length, including the length of any side branches, was then divided by the number of branches to provide information about the peripheral growth unit (PGU) length, which is indicative of changes in hyphal morphology (Vesentini *et al.*, 2006b).

**2.7 Statistical analysis**

The daily rate of growth (see section 2.3) was calculated as:

$$\text{Rate of grown} = \frac{\sum((L / \Delta t)*24)}{n}$$

Where:

L - is the length between the inoculum and the terminal zone of the fungal colony

Δt – is the time of growth in hours

n – is the number of measurements

Changes in radial growth on different growth media and during anaerobiotic growth were evaluated. Putative differences of growth where then analysed using Anova. The comparisons were made among fungal colonies of the same species. The different analyses were made to detect differences among presence of $O_2$, absence of $O_2$ and phase post-anaerobiosis. For fungi that grew on different media (see section 2.6), the same formula was used to calculate the daily growth rate, and calculations were done for the fourth and eighth days.

The percentage of wood loss was calculated using the formula

$$\text{Loss wood (\%)} = \frac{\sum((m_i-m_f)/m_i)*100}{N}$$

Where:

$m_i$ – is the initial mass

$m_f$ – is the final mass

n – is the number of measurements

Variations in hyphal morphology were analysed by Anova. The average and standard deviation of the hyphae length was calculated, to register putative differences in fungal development.

All calculations were made with the Microsoft Excel program.

# 3. RESULTS

### 3.1. Effect of oxygen availability and temperature on fungal growth rate

All fungal species under analysis, *Trametes versicolor*, *Fomitiporia mediterranea*, and *Phaeomoniella chlamydospora,* achieved visible growth in Petri dishes containing PDA medium under aerobic conditions. However, the fungi exposed to anaerobic conditions were unable to grow, until oxygen deprivation had stopped. When oxygen was made available, growth resumed in a fashion comparable to that of the control cultures.

In Figures 3.1, 3.2, 3.3, 3.4 and 3.5, the morphological characteristics of the fungal colonies incubated under aerobic, anaerobic and aerobic post-anaerobic conditions are shown. Apart from differences in the growth rate, no other visible differences were detected in the fungi. During anaerobic growth, at both temperatures tested (15 and 24 ºC) no growth was detected among the three fungal species under analysis.

*Trametes versicolor* was overall the fastest growing fungus, whilst *Phaeomoniella chlamydospora* was the slowest one.

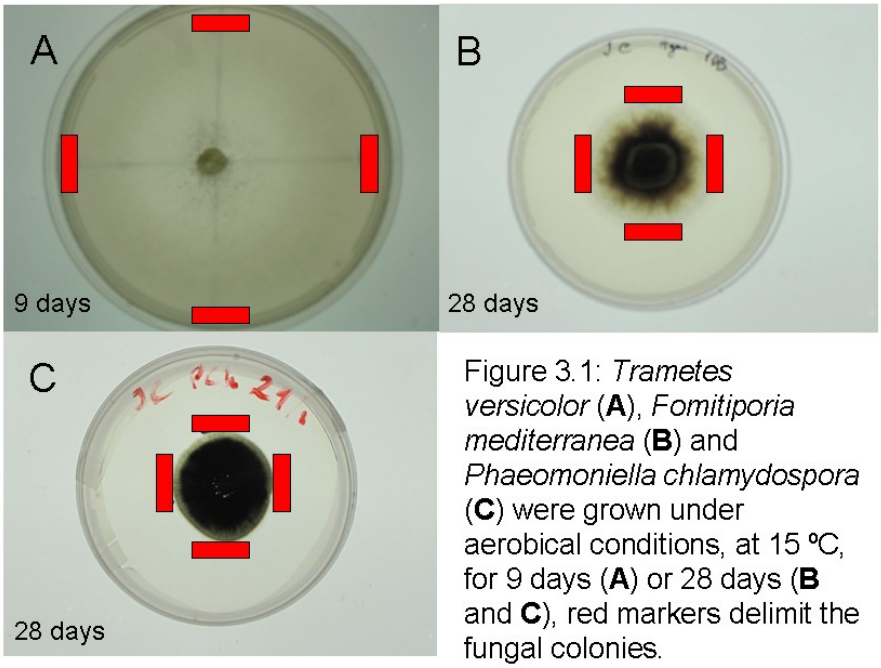


Figure 3.1: *Trametes versicolor* (**A**), *Fomitiporia mediterranea* (**B**) and *Phaeomoniella chlamydospora* (**C**) were grown under aerobical conditions, at 15 ºC, for 9 days (**A**) or 28 days (**B** and **C**), red markers delimit the fungal colonies.

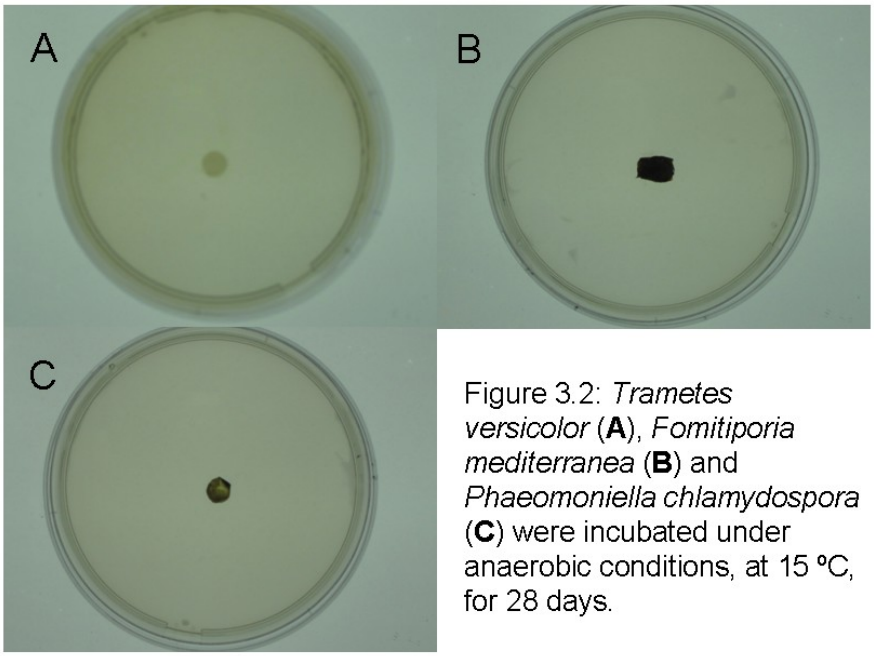


Figure 3.2: *Trametes versicolor* (**A**), *Fomitiporia mediterranea* (**B**) and *Phaeomoniella chlamydospora* (**C**) were incubated under anaerobic conditions, at 15 ºC, for 28 days.

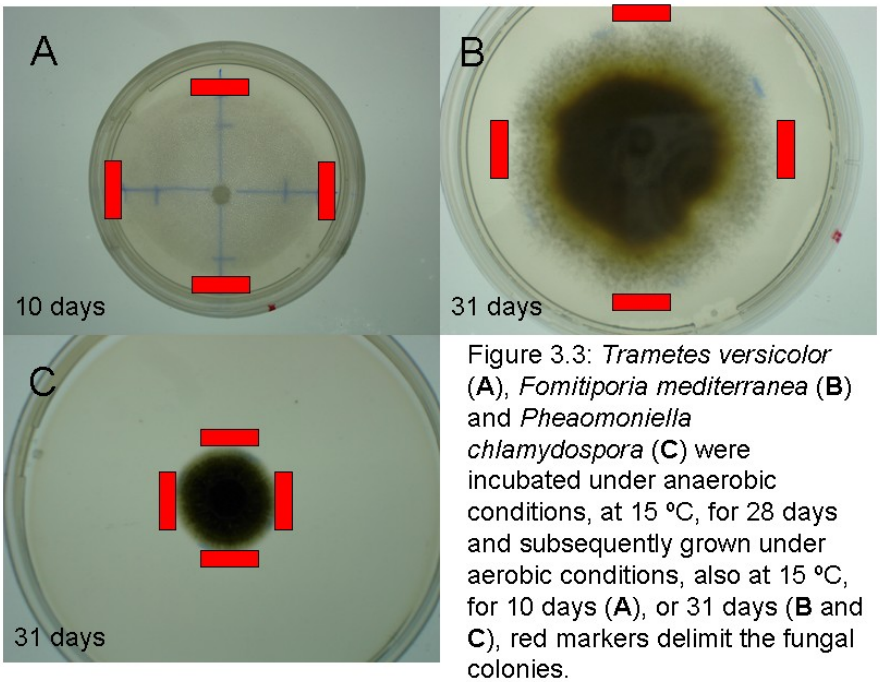


Figure 3.3: *Trametes versicolor* (**A**), *Fomitiporia mediterranea* (**B**) and *Pheaomoniella chlamydospora* (**C**) were incubated under anaerobic conditions, at 15 ºC, for 28 days and subsequently grown under aerobic conditions, also at 15 ºC, for 10 days (**A**), or 31 days (**B** and **C**), red markers delimit the fungal colonies.

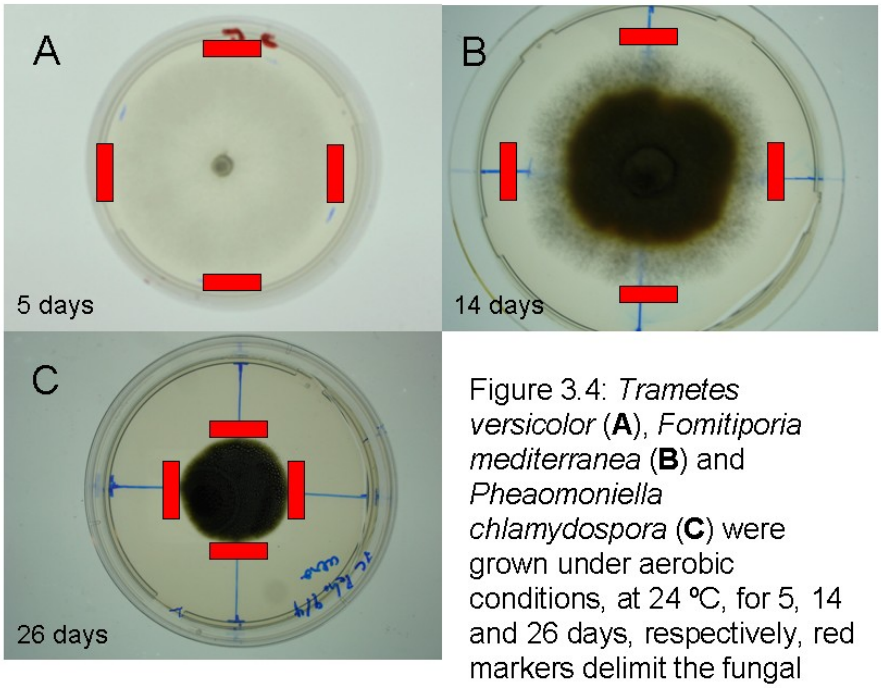


Figure 3.4: *Trametes versicolor* (**A**), *Fomitiporia mediterranea* (**B**) and *Pheaomoniella chlamydospora* (**C**) were grown under aerobic conditions, at 24 ºC, for 5, 14 and 26 days, respectively, red markers delimit the fungal colonies.

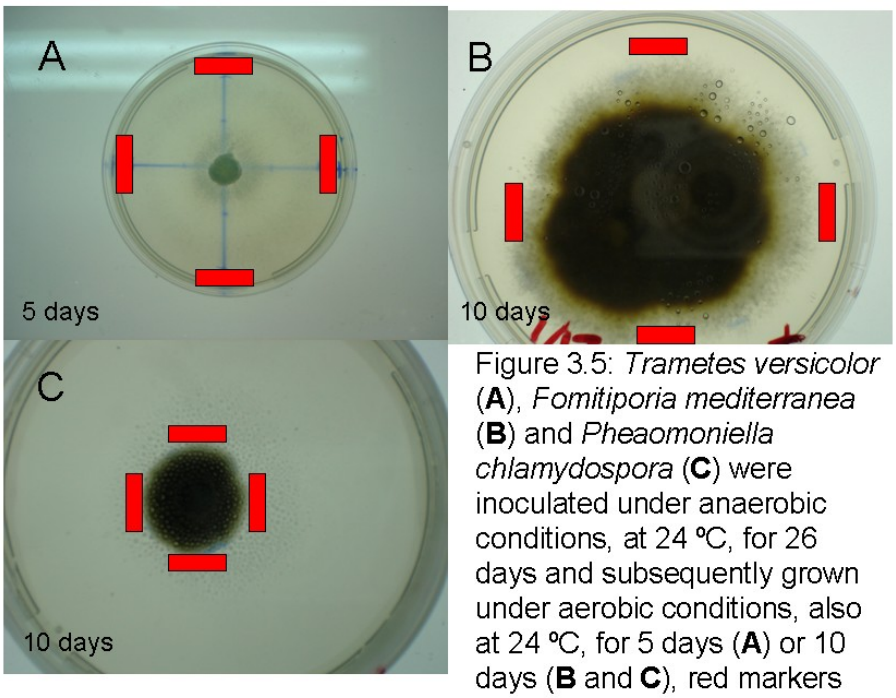


Figure 3.5: *Trametes versicolor* (**A**), *Fomitiporia mediterranea* (**B**) and *Pheaomoniella chlamydospora* (**C**) were inoculated under anaerobic conditions, at 24 ºC, for 26 days and subsequently grown under aerobic conditions, also at 24 ºC, for 5 days (**A**) or 10 days (**B** and **C**), red markers delimit the fungal colonies.

In Figure 3.6 it is possible to observe the effect of temperature on fungal growth under aerobic conditions versus anaerobic conditions. At 15 ºC *T. versicolor* grew at a rate of 3.598 mm $day^{-1}$ and under aerobic post-anaerobic conditions at a rate of 2.928 mm $day^{-1}$. The p-value is $2.32x10^{-8}$ meaning that this difference is significant. As observed before, no growth was detected under anaerobic conditions. At 24 ºC, under aerobic conditions, the growth rate was 6 mm $day^{-1}$, whilst in aerobic post-anaerobic conditions was 6.167 mm $day^{-1}$. The respective p-value is 0.089584, meaning that this difference is not statistically significant. Although *T. versicolor* grew at a reduced rate at 15 ºC following exposure to anaerobic conditions, this did not occur at 24 ºC, where no significant difference in the rate of growth was observed in relation to the control.

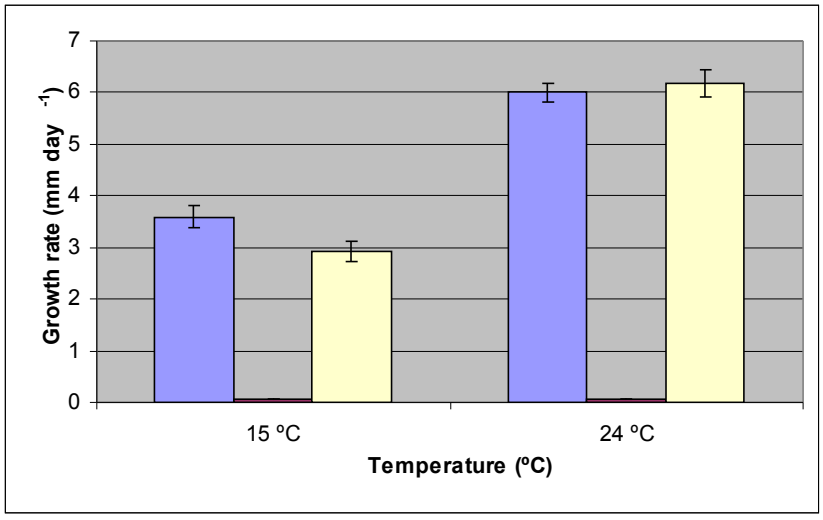


Figure 3.6: Growth rate expressed as the rate of increase of the colony diameter (mm $day^{-1}$) of *Trametes versicolor*, at 15 and 24 ºC, under aerobic conditions (**Blue**), under anaerobic conditions (**Red**) and under aerobic post-anaerobic phase (**Yellow**), with the respective standard deviation values.

Figure 3.7 shows the rate of growth of *F. mediterranea* at 15 ºC under aerobic conditions versus anaerobic. Under these conditions, the rate of growth was 0.491 mm $day^{-1}$, whilst under aerobic post-anaerobic conditions fungal growth was 0.634 mm $day^{-1}$. The p-value is 0.01342, indicating that this difference in growth is significant. At 24 ºC under aerobiotic conditions, *F. mediterranea* grew 2.101 mm $day^{-1}$, whilst aerobic post-anaerobic growth attained 2.658 mm $day^{-1}$; the p-value is $2.9x10^{-7}$, meaning that this difference is significant. Overall, *F. mediterranea* grew faster at both temperatures tested, following a previous exposure to anaerobiosis.

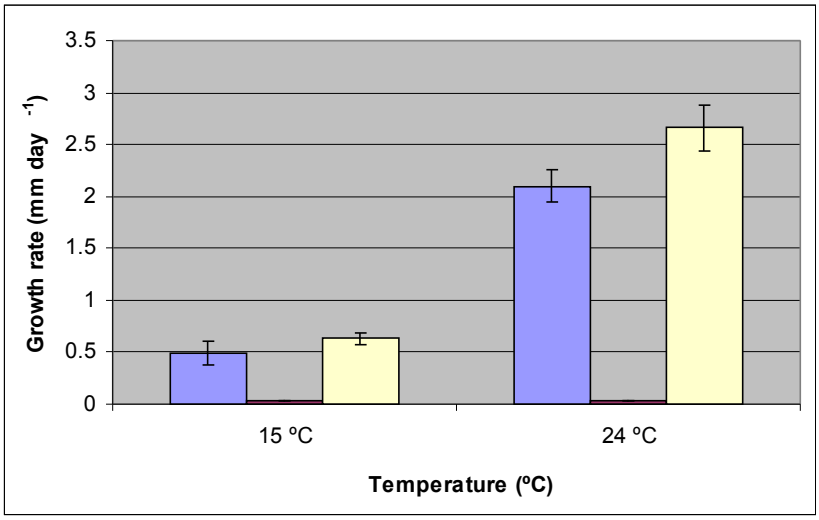


Figure 3.7: Growth rate expressed as the rate of increase of the colony diameter (mm $day^{-1}$) of *Fomitiporia mediterranea*, at 15 and 24 ºC, under aerobic conditions (**Blue**), under anaerobic conditions (**Red**) and under aerobic post-anaerobic conditions phase (**Yellow**), with the respective standard deviation values.

Figure 3.8 shows the rate of growth of *P. chlamydospora* in the presence and absence of oxygen. At 15 ºC, under aerobic conditions, the fungus attained a growth rate of 0.25 mm $day^{-1}$, whilst in aerobic post-anaerobic growth a rate of 0.253 mm $day^{-1}$ was observed. The p-value is 0.877245, meaning that this difference is not statistically significant. At 24 ºC, *P.*

*chlamydospora* grew at a rate of 0.664 mm day$^{-1}$, in the absence of oxygen limitation. Under aerobic post-anaerobic conditions, however, the rate of growth was 0.709 mm day$^{-1}$; p-value of 0.08013, a difference considered as not significant.

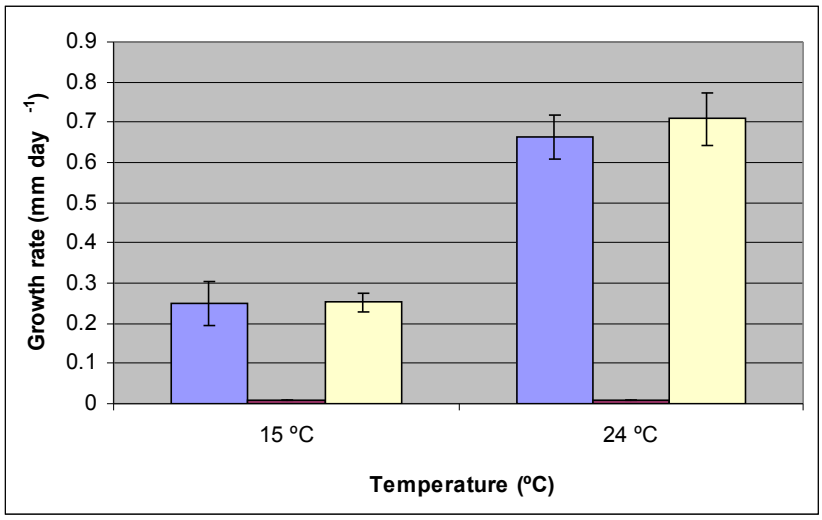


Figure 3.8: Growth rate expressed as the rate of increase of the colony diameter (mm day$^{-1}$) of *Pheaomoniella chlamydospora*, at 15 and 24 ºC, under aerobic conditions (**Blue**), in anaerobic conditions (**Red**) and under aerobic post-anaerobic conditions phase (**Yellow**), with the respective standard deviation values.

### 3.2. Fungal growth evaluation

Fungal growth (*Trametes versicolor*, *Fomitiporia mediterranea*, and *Phaeomoniella chlamydospora*) was measured over time to assess whether any significant differences in the growth pattern of the fungi could be observed along the period of study. The results presented in Figure 3.9 indicate that the initial growth was slow for all of the fungi tested, but increased after the initial four days of growth.

The initial growth rate for *T. versicolor* was 5.75 mm day$^{-1}$ on the fourth day, whilst on the fifth day was 6.167 mm day$^{-1}$. The p-value is 0.001, meaning that the difference of growth is statistically significant. A similar pattern was observed for *F. mediterranea,* with 0.708 mm

day$^{-1}$ during the fourth day of growth and 1.229 mm day$^{-1}$ during the eighth day, with a p-value of 0.001. On *P. chlamydospora*, growth rate on the fourth day was 0.114 mm day$^{-1}$ and 0.438 mm day$^{-1}$ on the eighth day, with a p-value of 4.528x10$^{-10}$. In all cases differences in growth where found to be statistically significant.

During the first 8 days, *F. mediterranea* grew at rate of 1.229 mm day$^{-1}$, but this value increased to 2.101 mm day$^{-1}$ when 10 days of growth were analysed (p-value of 7.461x10$^{-10}$, meaning a statistical significant difference). In what *P. chlamydospora* is concerned, the growth rate was 0.438 mm day$^{-1}$ during the first 8 days, but increased to 0.664 mm day$^{-1}$ thereafter, with a p-value of 9.753x10$^{-9}$, meaning a statistically significant difference.

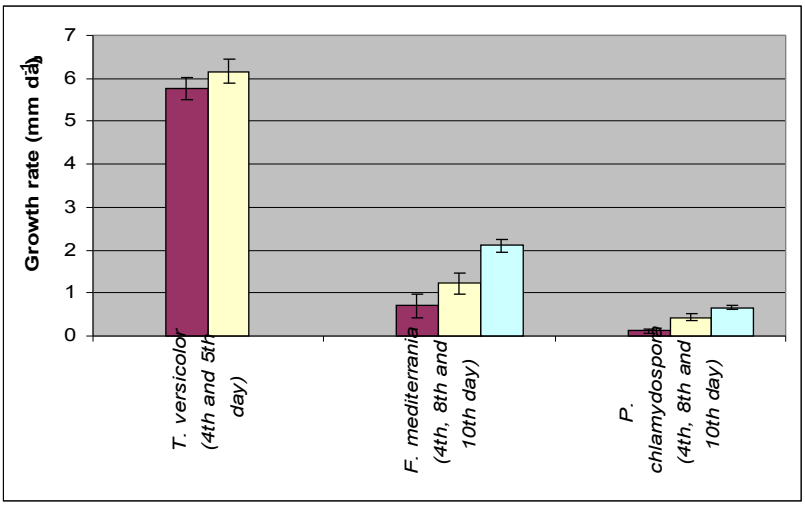


Figure 3.9: Growth rate of fungi at 24 ºC under aerobic conditions in 3 phases. The firsth phase (**Red**) started on the 1st day and finished on the 4th day to all fungi.,The second phase (**Yellow**) started on the 4th day and finished on the 5th day to the fungi *Trametes versicolor*, to the other fungi, the phase started on the 4th day and finished on the 8th day. The third phase (**Blue**) started on the 8th day and finished on the 10th day, notice that the fungus *Trametes versicolor* do not have a third phase, because it reached to the end of the plate.

### 3.3. Detection of reactive oxygen species in isolated or interacting fungal colonies.

The figure 3.10 shows the results obtained with isolated fungal colonies to detect superoxide radical ($O_2^-$). The blue marks show the concentration of superoxide ion, the higher the concentration, the darker the blue marker. Figure 3.11 shows results obtained with interacting fungal colonies for detection of superoxide. It is possible to see one intense mark between *T. versicolor* and *F. mediterranea* (**A**) or between *T. versicolor* and *P. chlamydospora* (**B**), which indicate high levels of superoxide. Between *F. mediterranea* and

*P. chlamydospora* (**C**) no intense mark was observed, which indicates the presence of low levels of superoxide ion.

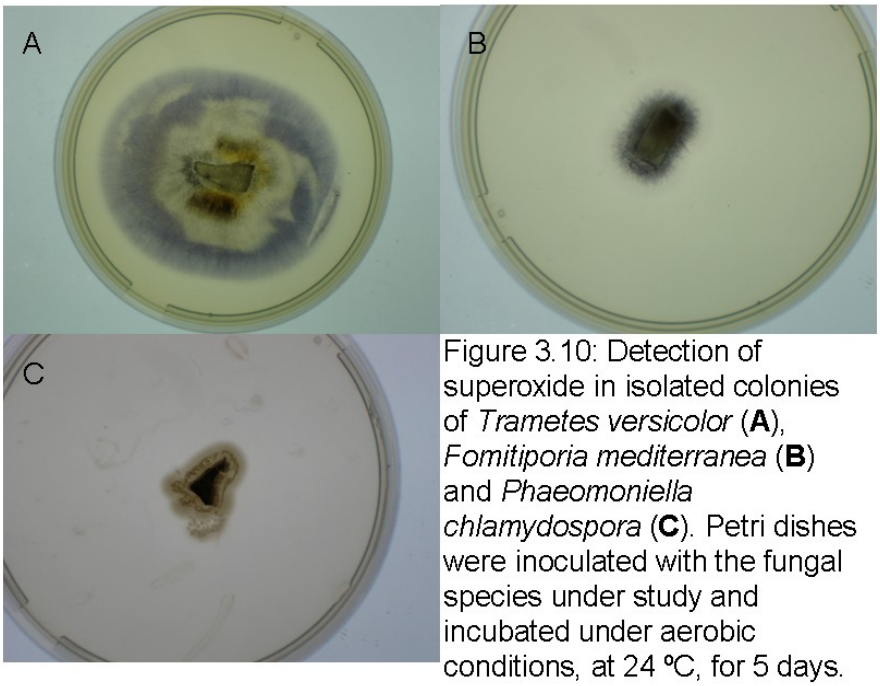


Figure 3.10: Detection of superoxide in isolated colonies of *Trametes versicolor* (**A**), *Fomitiporia mediterranea* (**B**) and *Phaeomoniella chlamydospora* (**C**). Petri dishes were inoculated with the fungal species under study and incubated under aerobic conditions, at 24 ºC, for 5 days.

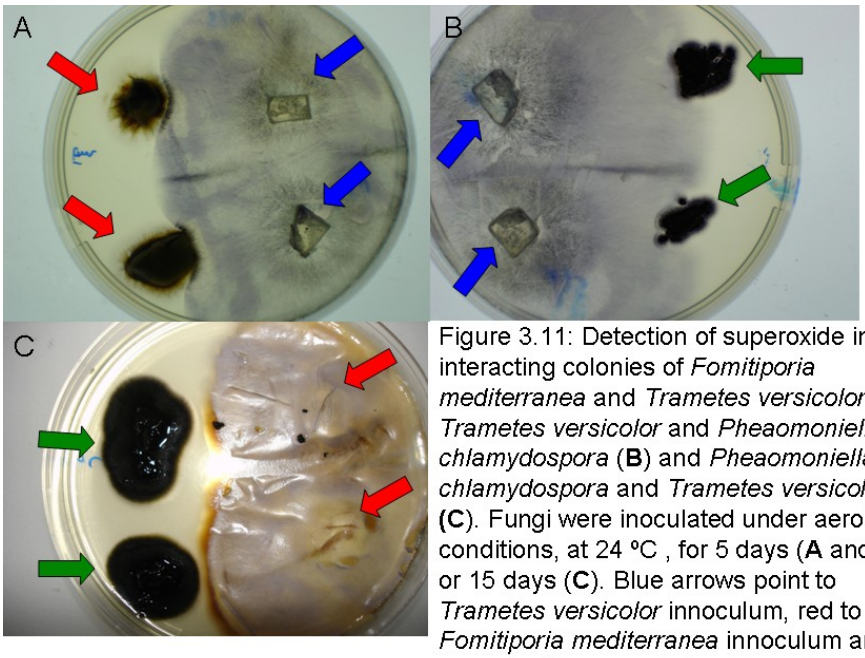


Figure 3.11: Detection of superoxide in interacting colonies of *Fomitiporia mediterranea* and *Trametes versicolor* (**A**), *Trametes versicolor* and *Pheaomoniella chlamydospora* (**B**) and *Pheaomoniella chlamydospora* and *Trametes versicolor* (**C**). Fungi were inoculated under aerobic conditions, at 24 ºC , for 5 days (**A** and **B**), or 15 days (**C**). Blue arrows point to *Trametes versicolor* innoculum, red to *Fomitiporia mediterranea* innoculum and green to *Pheaomoniella chlamydospora* innoculum.

Figure 3.12 shows the results achieved for the isolated fungal species under study to detect hydrogen peroxide ($H_2O_2$). In the methodology used (see materials and methods) the reagent turns yellow where the hydrogen peroxide is more concentrated. Figure 3.13 illustrate the results obtained for the interacting fungal colonies for detection of $H_2O_2$. It is possible to see the production of hydrogen peroxide; yellow marks are more intense in the interactions of *T. versicolor* with esca fungi, then with esca fungi interacting among themselves.

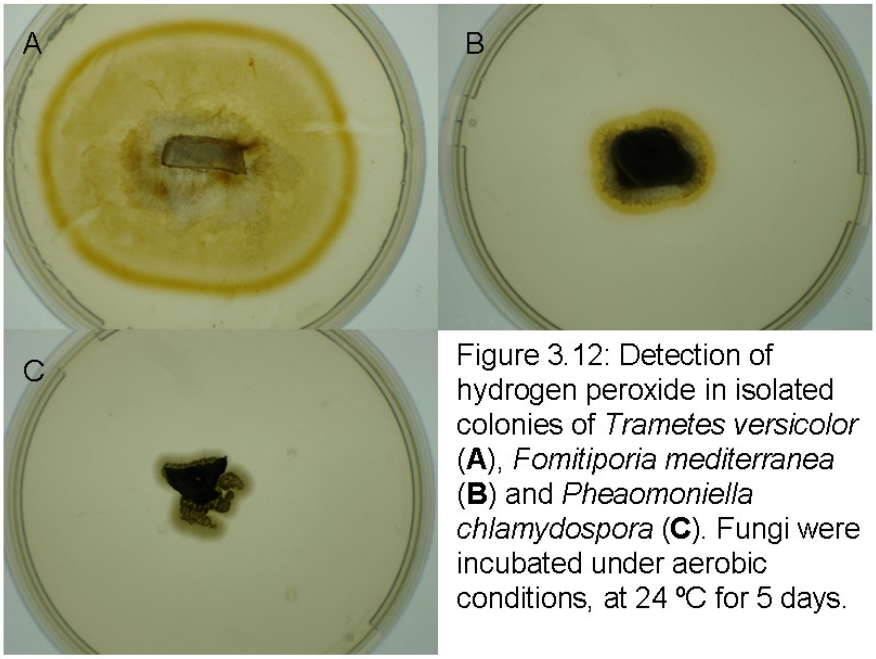


Figure 3.12: Detection of hydrogen peroxide in isolated colonies of *Trametes versicolor* (**A**), *Fomitiporia mediterranea* (**B**) and *Pheaomoniella chlamydospora* (**C**). Fungi were incubated under aerobic conditions, at 24 ºC for 5 days.

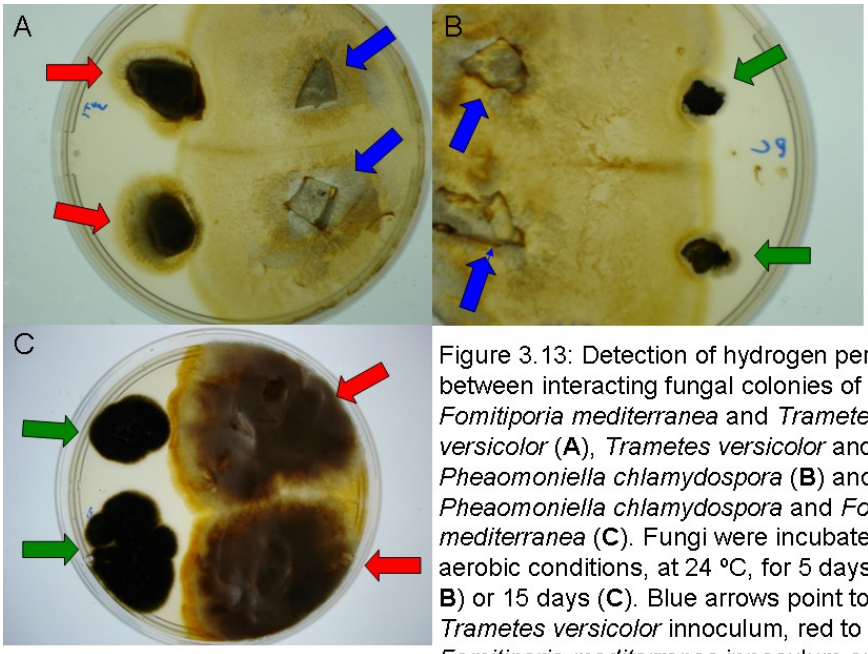


Figure 3.13: Detection of hydrogen peroxide between interacting fungal colonies of *Fomitiporia mediterranea* and *Trametes versicolor* (**A**), *Trametes versicolor* and *Pheaomoniella chlamydospora* (**B**) and *Pheaomoniella chlamydospora* and *Fomitiporia mediterranea* (**C**). Fungi were incubated under aerobic conditions, at 24 ºC, for 5 days (**A** and **B**) or 15 days (**C**). Blue arrows point to *Trametes versicolor* innoculum, red to *Fomitiporia mediterranea* innoculum and green to *Pheaomoniella chlamydospora* innoculum.

### 3.4 Test wood degradation

Four plastic rings were placed inside Petri dishes containing PDA, which were subsequently inoculated with the fungal species under study, *Trametes versicolor*, *Fomitiporia mediterranea*, and *Phaeomoniella chlamydospora*. Then, blocks of wood (*Prunus* spp, *Ficus carica*, *Vitis vinifera*) were placed onto the plastic rings. The fungi were allowed to grow and the hyphae invaded the wood. In the case of *T. versicolor* and *F. mediterranea* the wood was totally surrounded by one layer of fungal hyphae, but the layer of *F. mediterranea* was thicker than the layer of *T. versicolor*. The wood invaded by *T. versicolor* and by *F. mediterranea* had a spongy consistency, especially in the case of the wood of *Ficus carica*. In addition, the wood of *V. vinifera* infected by those fungi changed colour from brown to black, as if it had been burnt. In the case of *P. chlamydospora*, a complete layer could not be established around the wooden block since this fungus exhibits a low rate of growth. Instead, only the surface below of the wooden block was invaded by hyphae. The results presented in figure 3.14 show the extent of wood degradation after a 38-day incubation with the fungal species under study. The differences that are significant ($p\text{-value}<0.05$ and $p\text{-value}\geq0.01$) are marked with *; the differences highly significant ($p\text{-value}<0.01$) are marked with **; and the differences not significant ($p\text{-value}>0.05$) were left unmarked.

The wood of *Prunus* exhibited 17.8% by weight of degradation with *T. versicolor*, 7.4% with *F. carica* and 3.0% witht *P. chlamydospora*. The wood of *F. carica* suffered 41.2, 13.4 and 4.2% of degradation with the fungi *T. versicolor*, *F. carica* and *P. chlamydospora,* respectively. In the case of *V. viniferawood degradation,* the values were 36.7% for *T. versicolor*, 11.5% for *F. mediterranea* and 4.7% for *P.chlamydospora*.

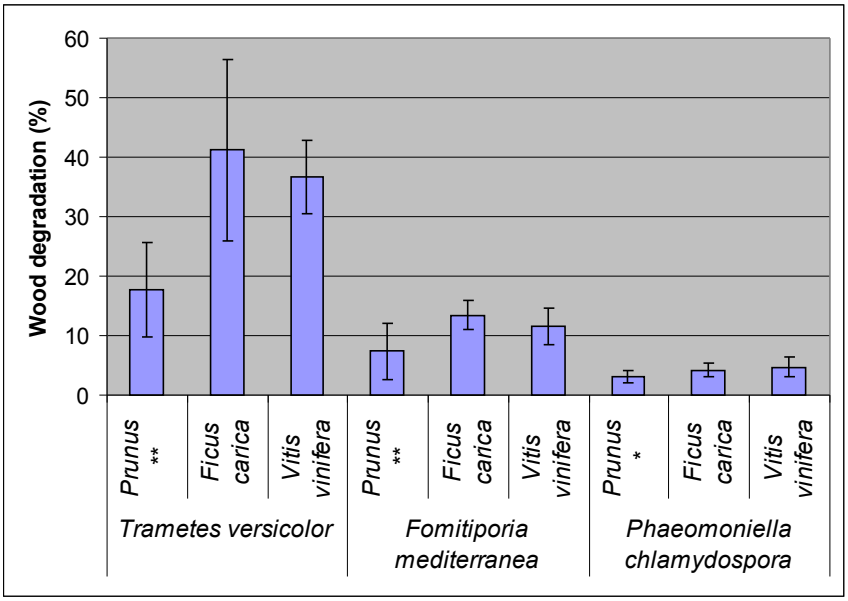


Figure 3.14: Degradation of *Prunus* spp *Ficus carica* and *Vitis vinifera* wood, after 38 days of infection by the fungal species under study. Fungi were grown under aerobic conditions at 24 ºC (n=10).

Figures 3.15 through 3.20 show details of the degraded woody substrate following growth of the individual fungi. *T. versicolor* (Figures 3.15 and 3.18: transversal and tangential sections, respectively) was the fungus that caused the most intense degradation on the three species of wood under study, a result which could be observed with the naked eye. *F. mediterranea* (Figures 3.16 and 3.19) caused some damage on all wood types. Finally, *P. chlamydospora* caused the smallest damage, the extent of which could only be observed in thin section preparations, as it was not visible using a stereomicroscope (Figures 3.17 and 3.20).

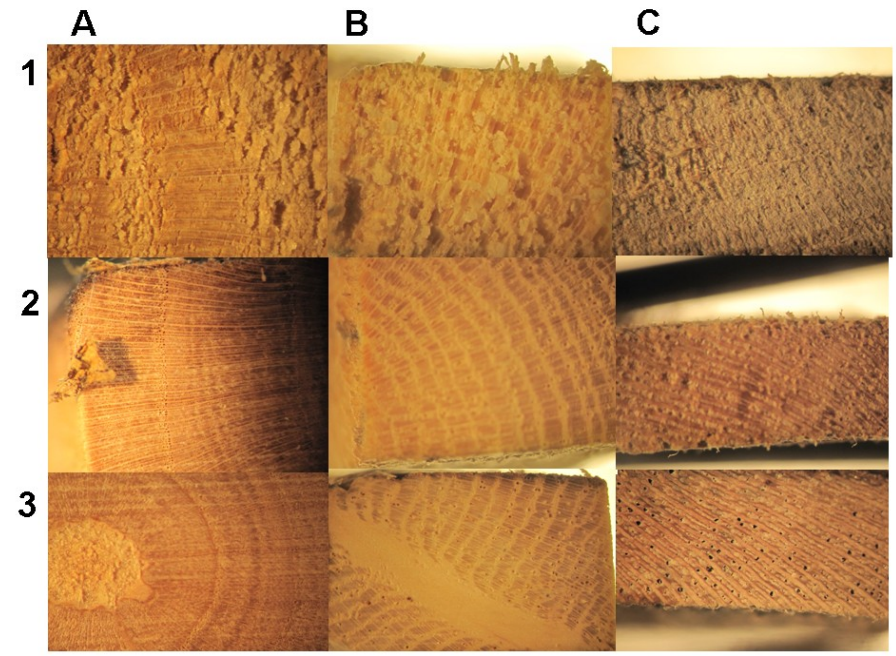


Figure 3.15: Tranversal sections of wood following interaction with the fungi. Letters identify the type of wood. Numbers identify the fungal specie. *Prunus* (A), *Ficus carica* (B), *Vitis vinifera* (C). *Trametes versicolor* (1), *Fomitiporia mediterranea* (2), *Phaeomoniella chlamydospora* (3).

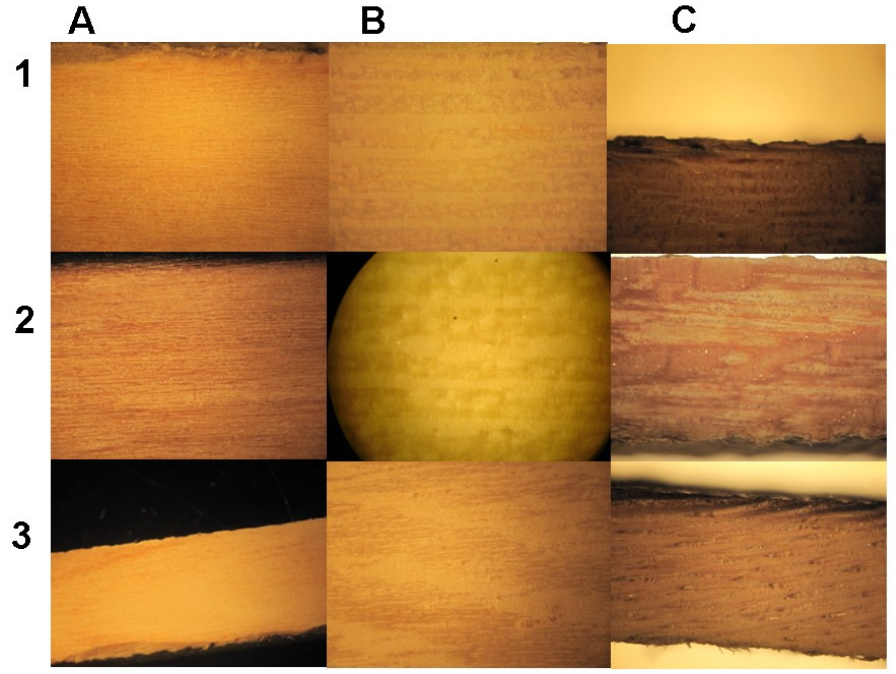


Figure 3.16: Tangential section of wood following interaction with fungi. Letters identify the type of wood. Numbers identify the fungal specie. *Prunus* (A), *Ficus carica* (B), *Vitis vinifera* (C). *Trametes versicolor* (1), *Fomitiporia mediterranea* (2), *Phaeomoniella chlamydospora* (3).

Histological observations were made to assess the type of damage that fungi caused in the cell walls. Wood was observed without stain (Figures 3.24, 3.25, 3.26, 3.27, 3.28 and 3.29) in order to have a general overview of the damage, without addressing specific cell wall components. The most prominent degradation was caused by *T. versicolor*, most notably in the wood of *Vitis vinifera*. *F. mediterranea* caused an intermediate level of damage in all types of wood, whilst *P chlamydospora* was the fungus that caused the least damage. In all cases, damage occurred principally in vessels and surrounding areas.

The phloroglucinol-HCl method allowed observation of damage to lignin, which is stained in red-violet following the histological preparation (Figures 3.30, 3.31, 3.32, 3.33, 3.34 and 3.35).

The methylene blue method allowed for observation of cellulose, which was stained blue following the histological preparation (Figures 3.36, 3.37, 3.38, 3.39, 3.40 and 3.41). In what cellulose is concerned, *T. versicolor was* able to degraded the polymer, mainly in xylem vessels and surrounding cells, *F. mediterranea* was able to degraded cellulose, especially in xylem vessels of *V. vinifera,* whilst *P. chlamydospora* caused limited damage to cellulose.

The picroaniline blue method used to observe fungal hyphae was associated with the development of a blue staining in the fungi, whilst the plant cell walls appeared pink (3.42, 3.43, 3.44, 3.45, 3.46 and 3.47). Hyphae of all fungi were visible in the xylem and surrounded cells of the three types of wood, especially in the lumen.

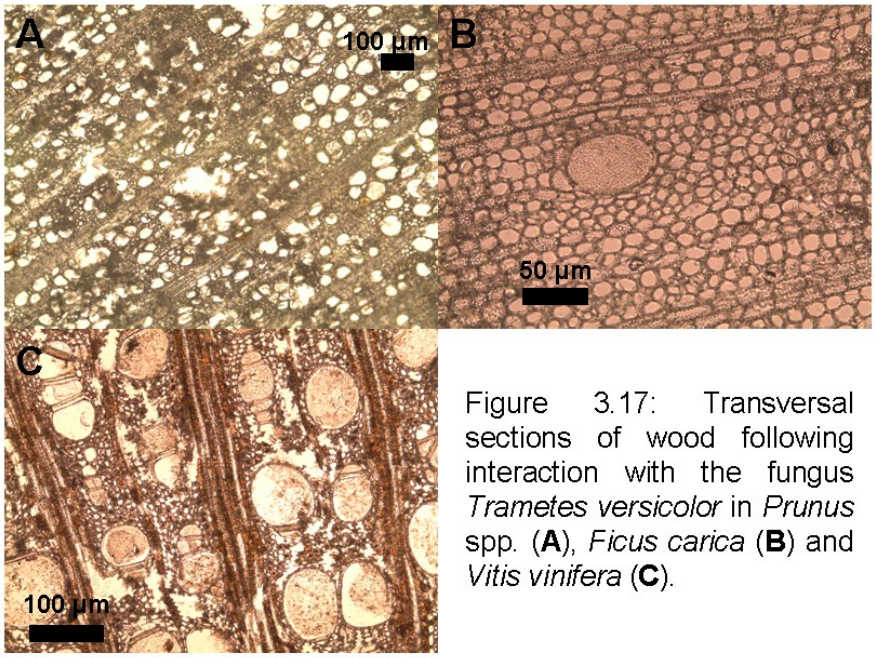


Figure 3.17: Transversal sections of wood following interaction with the fungus *Trametes versicolor* in *Prunus* spp. (**A**), *Ficus carica* (**B**) and *Vitis vinifera* (**C**).

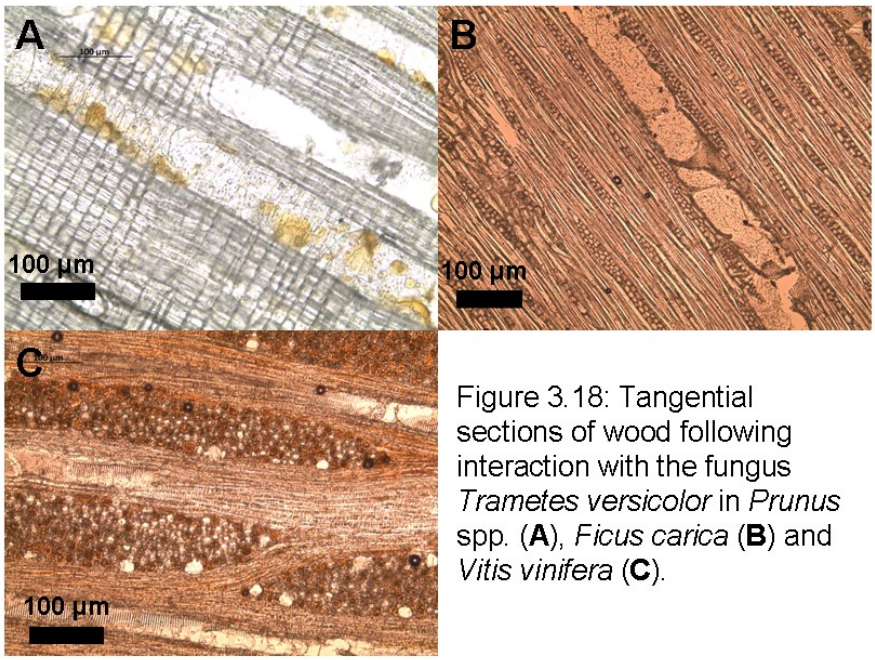


Figure 3.18: Tangential sections of wood following interaction with the fungus *Trametes versicolor* in *Prunus* spp. (**A**), *Ficus carica* (**B**) and *Vitis vinifera* (**C**).

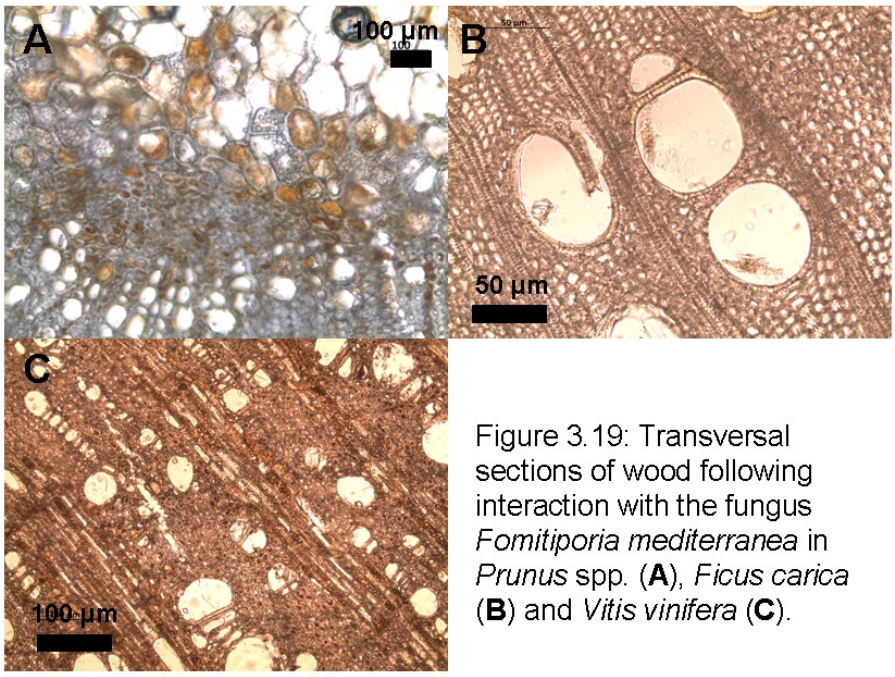


Figure 3.19: Transversal sections of wood following interaction with the fungus *Fomitiporia mediterranea* in *Prunus* spp. (**A**), *Ficus carica* (**B**) and *Vitis vinifera* (**C**).

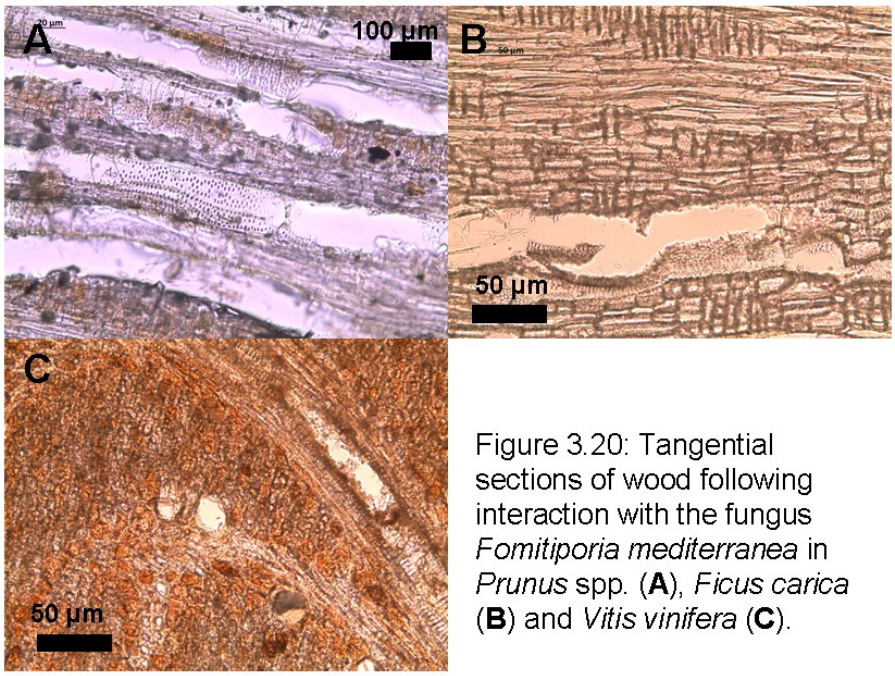


Figure 3.20: Tangential sections of wood following interaction with the fungus *Fomitiporia mediterranea* in *Prunus* spp. (**A**), *Ficus carica* (**B**) and *Vitis vinifera* (**C**).

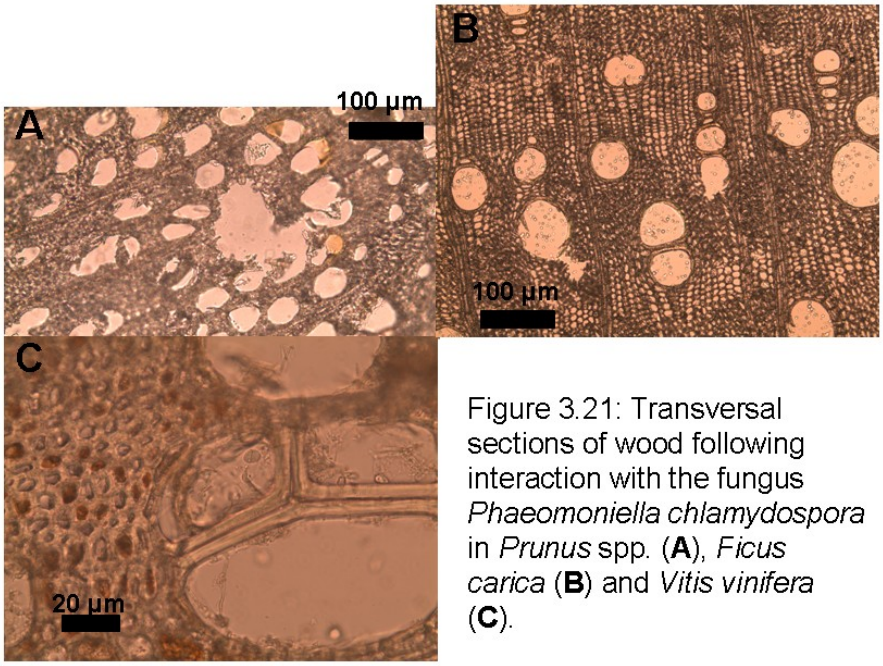


Figure 3.21: Transversal sections of wood following interaction with the fungus *Phaeomoniella chlamydospora* in *Prunus* spp. (**A**), *Ficus carica* (**B**) and *Vitis vinifera* (**C**).

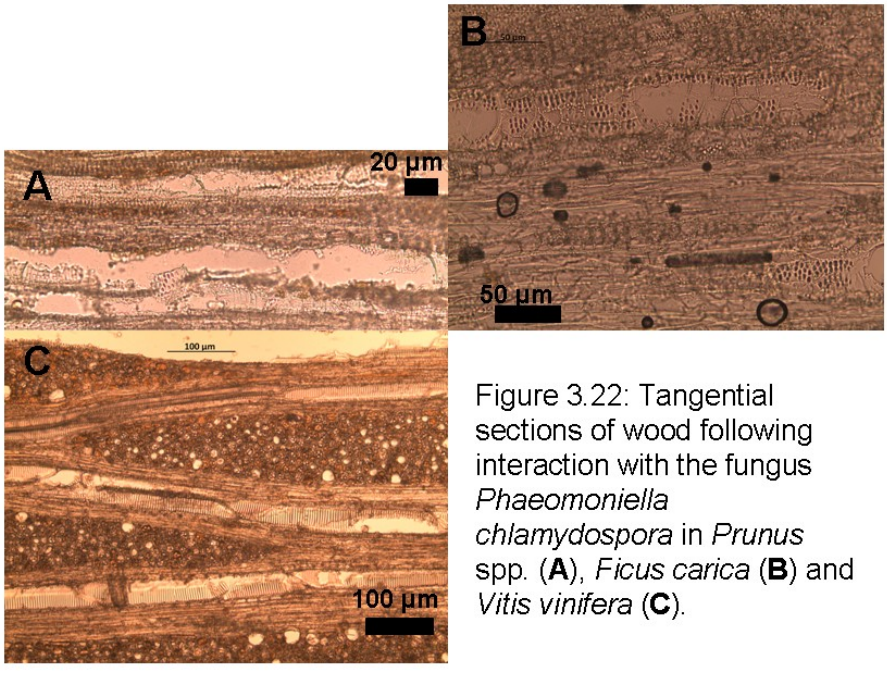


Figure 3.22: Tangential sections of wood following interaction with the fungus *Phaeomoniella chlamydospora* in *Prunus* spp. (**A**), *Ficus carica* (**B**) and *Vitis vinifera* (**C**).

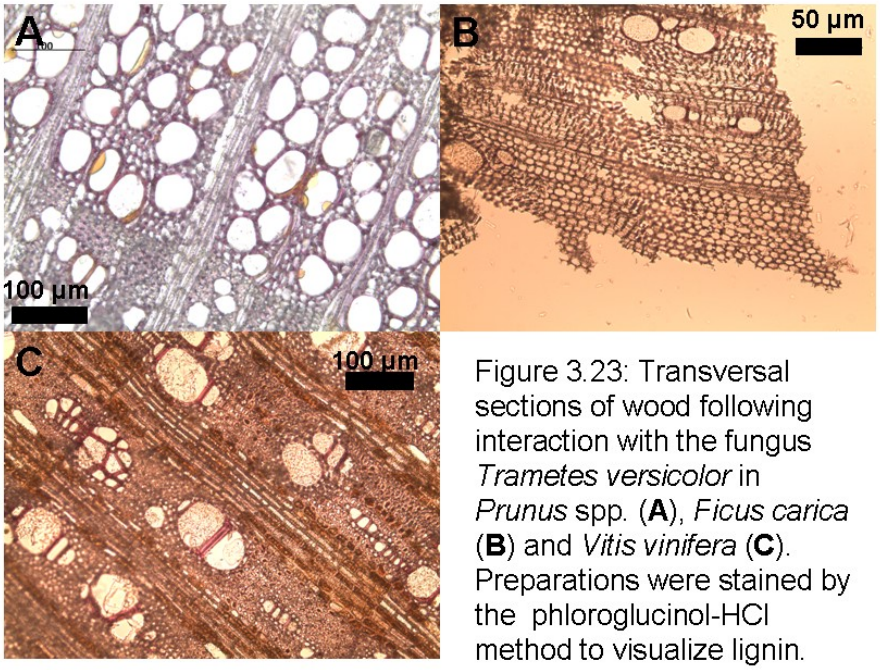


Figure 3.23: Transversal sections of wood following interaction with the fungus *Trametes versicolor* in *Prunus* spp. (**A**), *Ficus carica* (**B**) and *Vitis vinifera* (**C**). Preparations were stained by the phloroglucinol-HCl method to visualize lignin.

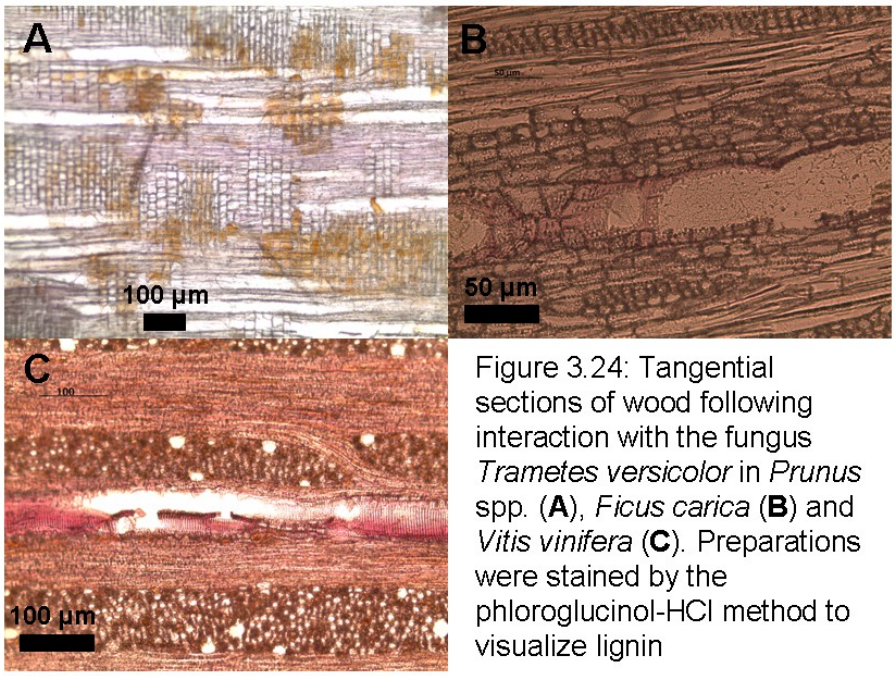


Figure 3.24: Tangential sections of wood following interaction with the fungus *Trametes versicolor* in *Prunus* spp. (**A**), *Ficus carica* (**B**) and *Vitis vinifera* (**C**). Preparations were stained by the phloroglucinol-HCl method to visualize lignin

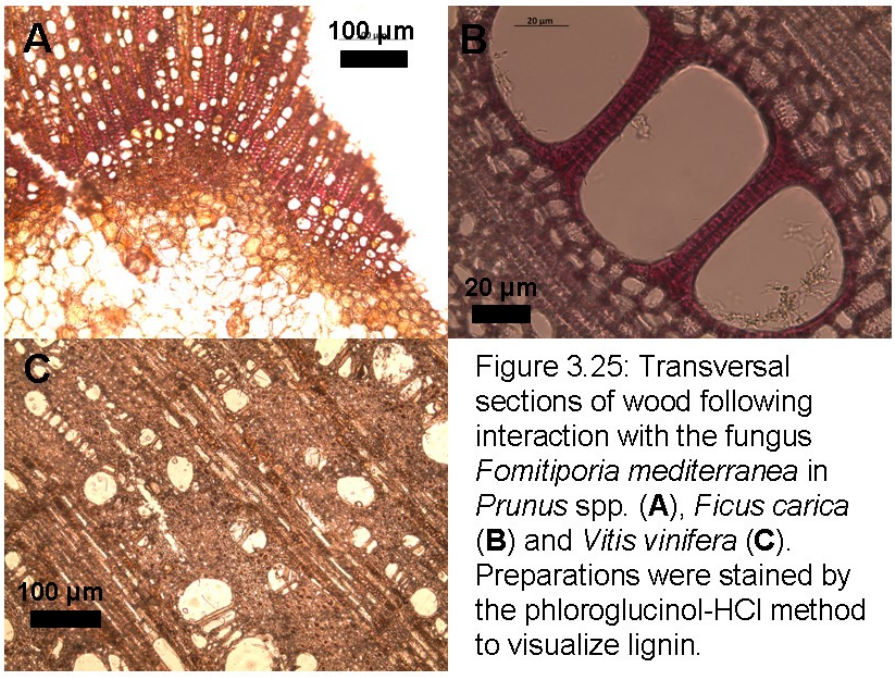


Figure 3.25: Transversal sections of wood following interaction with the fungus *Fomitiporia mediterranea* in *Prunus* spp. (**A**), *Ficus carica* (**B**) and *Vitis vinifera* (**C**). Preparations were stained by the phloroglucinol-HCl method to visualize lignin.

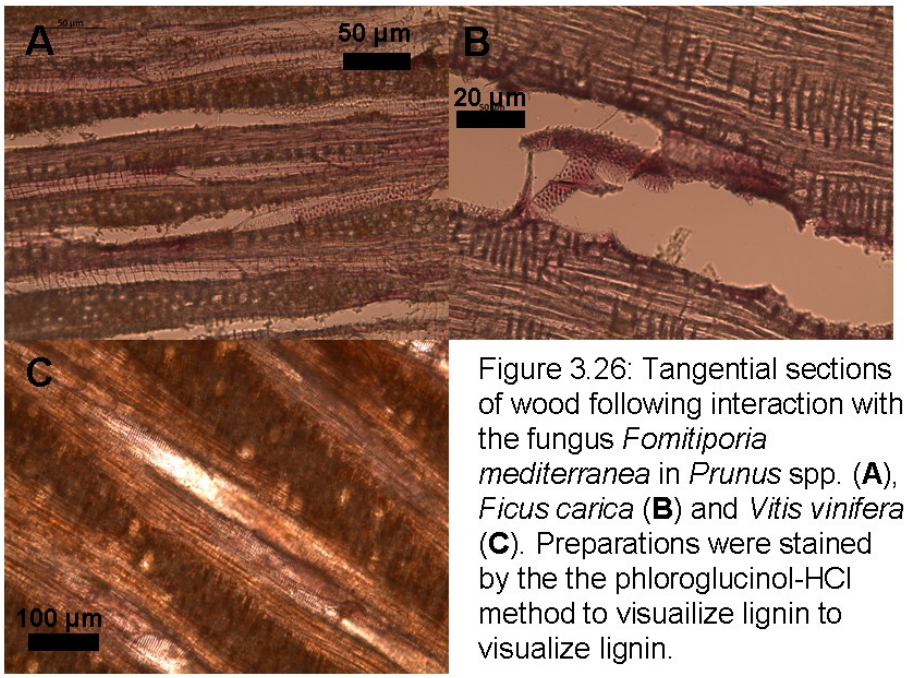


Figure 3.26: Tangential sections of wood following interaction with the fungus *Fomitiporia mediterranea* in *Prunus* spp. (**A**), *Ficus carica* (**B**) and *Vitis vinifera* (**C**). Preparations were stained by the the phloroglucinol-HCl method to visuailize lignin to visualize lignin.

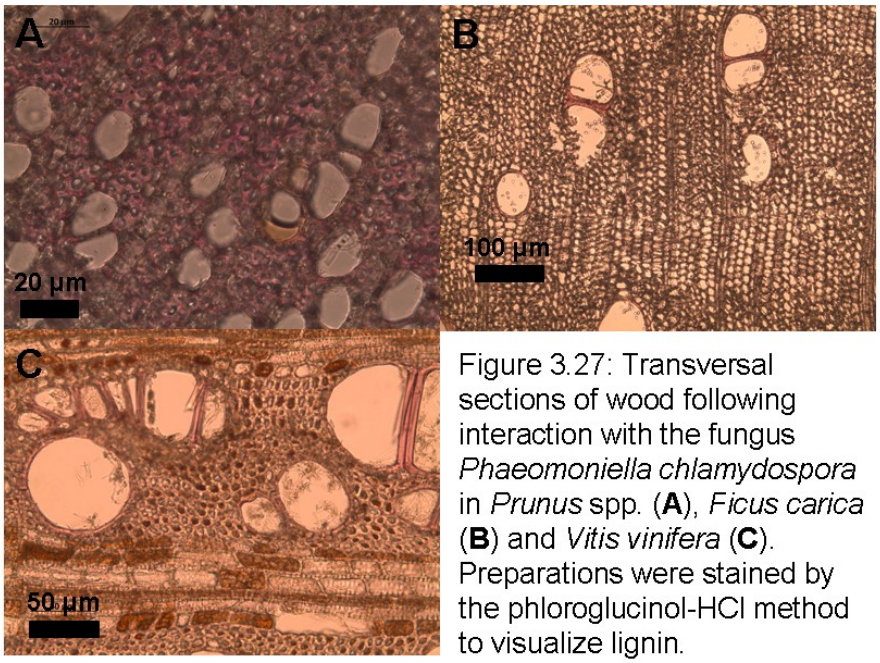


Figure 3.27: Transversal sections of wood following interaction with the fungus *Phaeomoniella chlamydospora* in *Prunus* spp. (**A**), *Ficus carica* (**B**) and *Vitis vinifera* (**C**). Preparations were stained by the phloroglucinol-HCl method to visualize lignin.

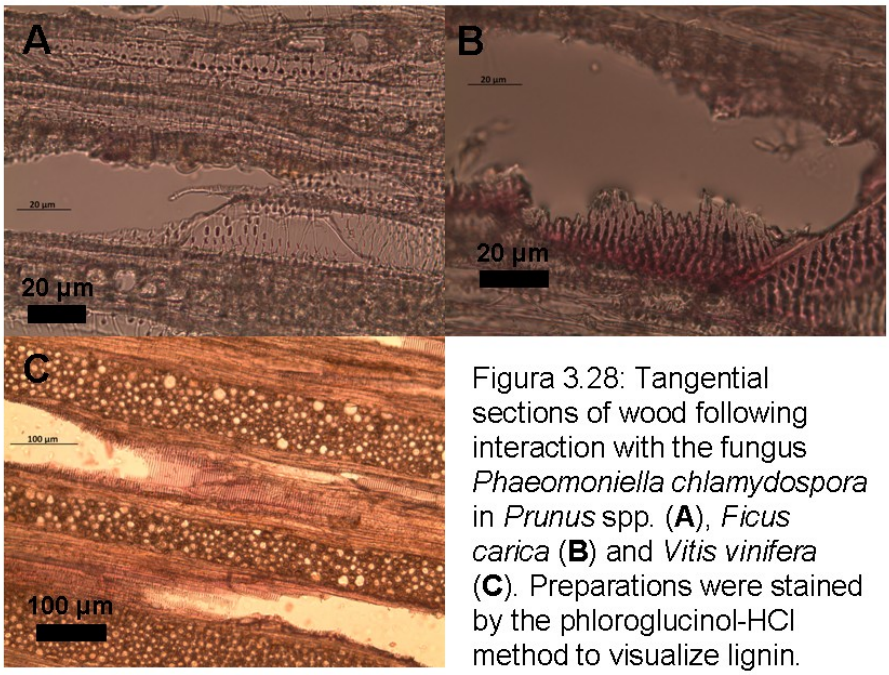


Figura 3.28: Tangential sections of wood following interaction with the fungus *Phaeomoniella chlamydospora* in *Prunus* spp. (**A**), *Ficus carica* (**B**) and *Vitis vinifera* (**C**). Preparations were stained by the phloroglucinol-HCl method to visualize lignin.

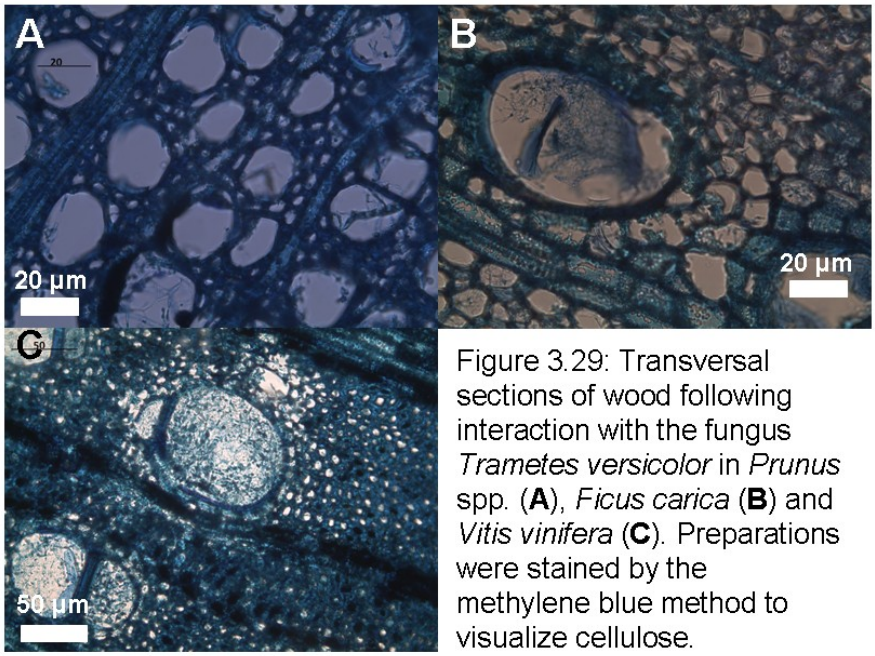


Figure 3.29: Transversal sections of wood following interaction with the fungus *Trametes versicolor* in *Prunus* spp. (**A**), *Ficus carica* (**B**) and *Vitis vinifera* (**C**). Preparations were stained by the methylene blue method to visualize cellulose.

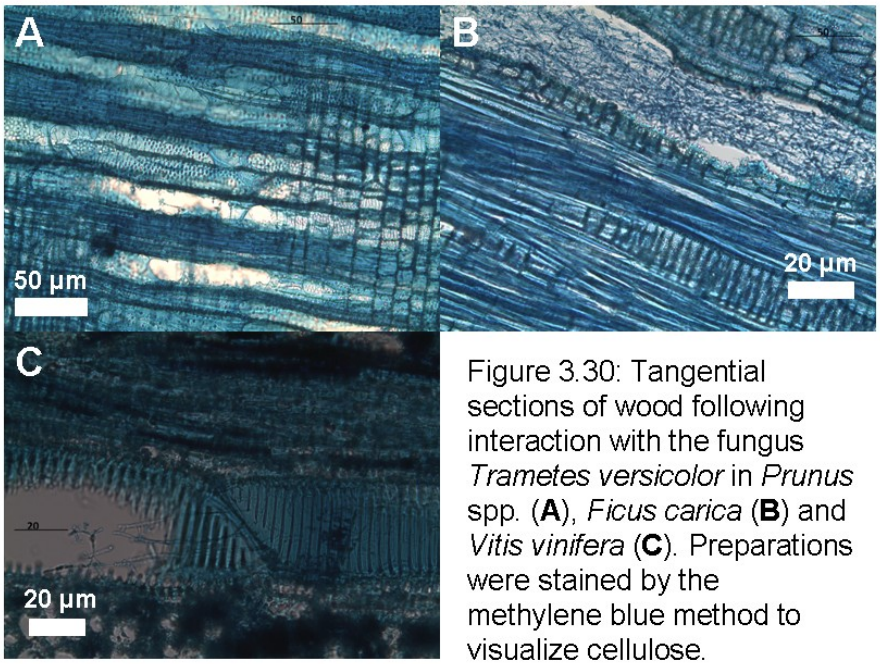


Figure 3.30: Tangential sections of wood following interaction with the fungus *Trametes versicolor* in *Prunus* spp. (**A**), *Ficus carica* (**B**) and *Vitis vinifera* (**C**). Preparations were stained by the methylene blue method to visualize cellulose.

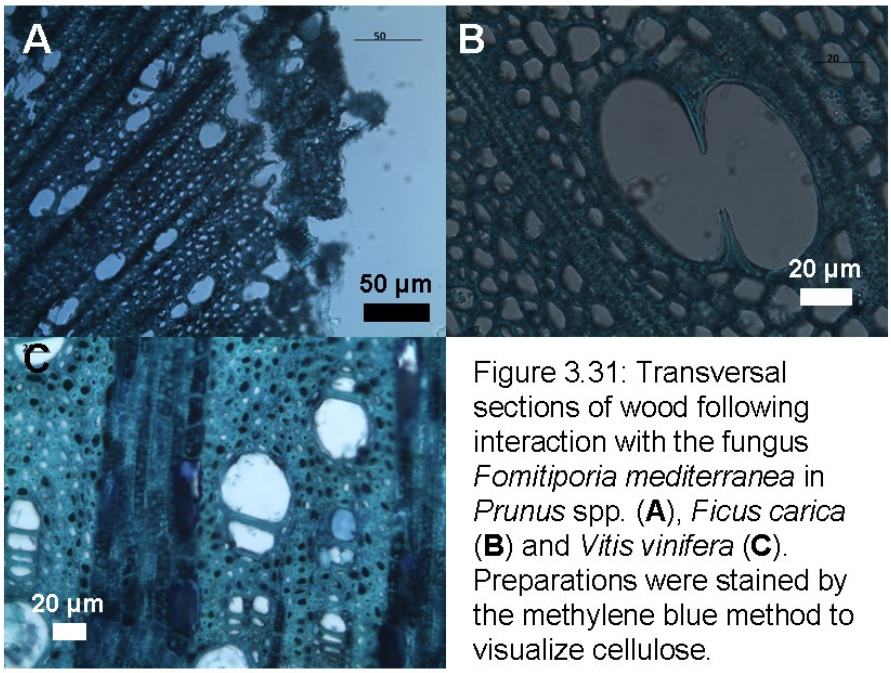


Figure 3.31: Transversal sections of wood following interaction with the fungus *Fomitiporia mediterranea* in *Prunus* spp. (**A**), *Ficus carica* (**B**) and *Vitis vinifera* (**C**). Preparations were stained by the methylene blue method to visualize cellulose.

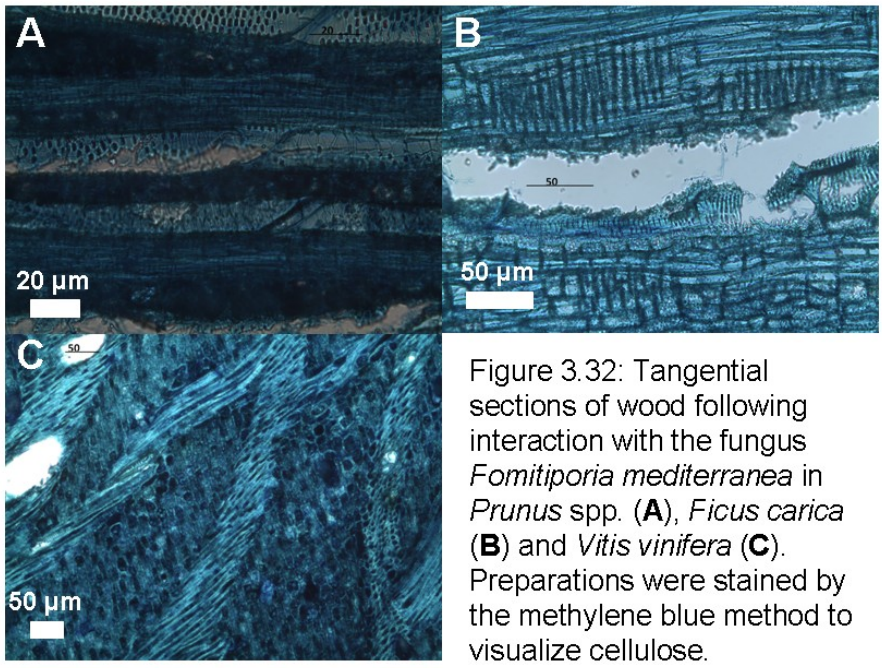


Figure 3.32: Tangential sections of wood following interaction with the fungus *Fomitiporia mediterranea* in *Prunus* spp. (**A**), *Ficus carica* (**B**) and *Vitis vinifera* (**C**). Preparations were stained by the methylene blue method to visualize cellulose.

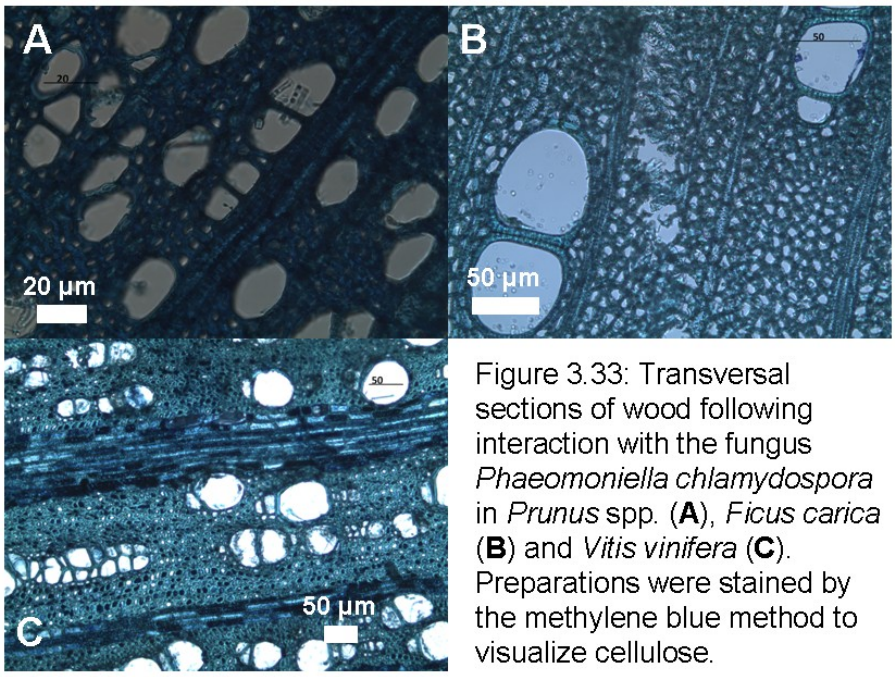


Figure 3.33: Transversal sections of wood following interaction with the fungus *Phaeomoniella chlamydospora* in *Prunus* spp. (**A**), *Ficus carica* (**B**) and *Vitis vinifera* (**C**). Preparations were stained by the methylene blue method to visualize cellulose.

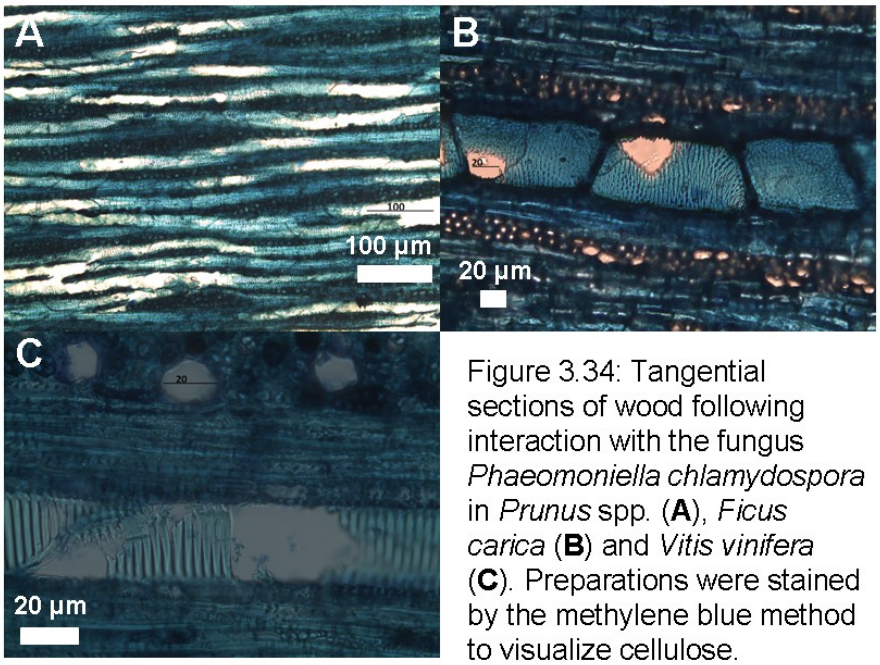


Figure 3.34: Tangential sections of wood following interaction with the fungus *Phaeomoniella chlamydospora* in *Prunus* spp. (**A**), *Ficus carica* (**B**) and *Vitis vinifera* (**C**). Preparations were stained by the methylene blue method to visualize cellulose.

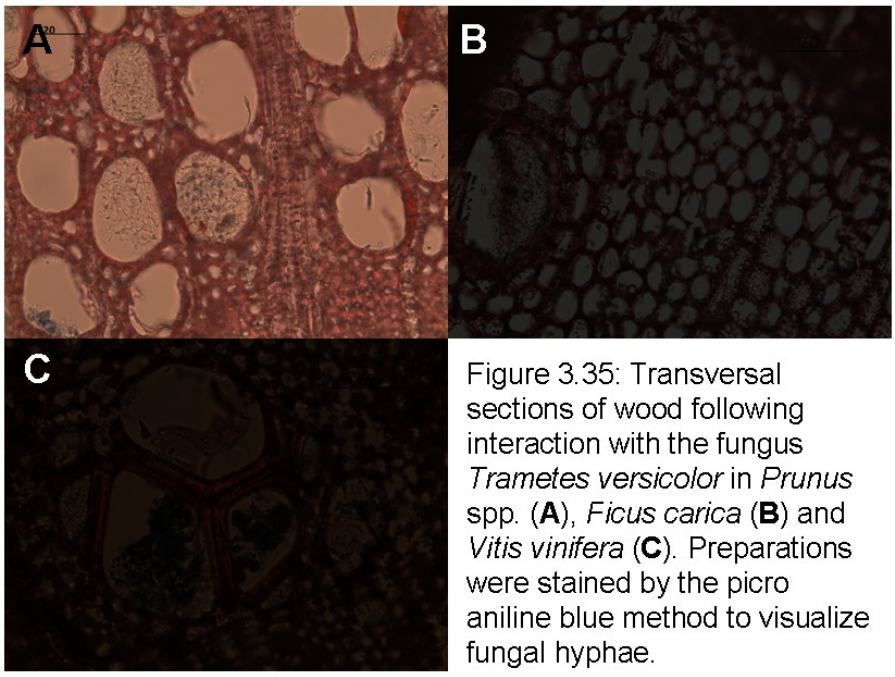


Figure 3.35: Transversal sections of wood following interaction with the fungus *Trametes versicolor* in *Prunus* spp. (**A**), *Ficus carica* (**B**) and *Vitis vinifera* (**C**). Preparations were stained by the picro aniline blue method to visualize fungal hyphae.

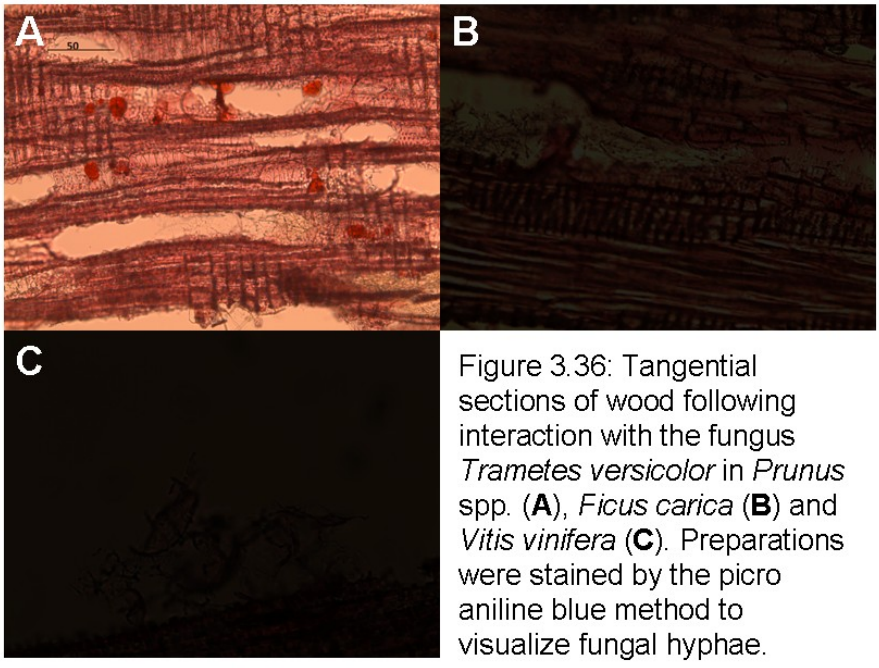


Figure 3.36: Tangential sections of wood following interaction with the fungus *Trametes versicolor* in *Prunus* spp. (**A**), *Ficus carica* (**B**) and *Vitis vinifera* (**C**). Preparations were stained by the picro aniline blue method to visualize fungal hyphae.

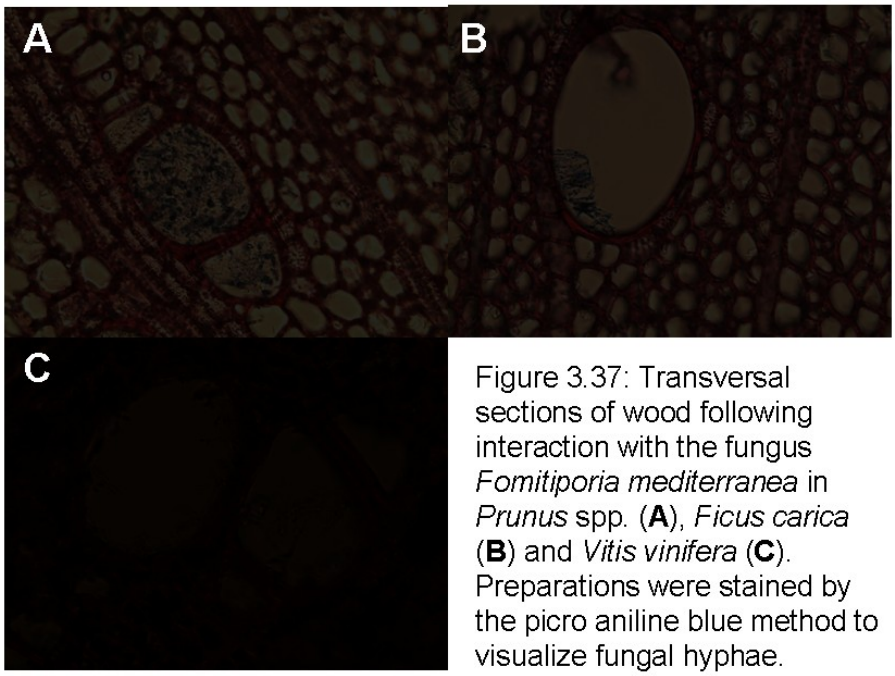


Figure 3.37: Transversal sections of wood following interaction with the fungus *Fomitiporia mediterranea* in *Prunus* spp. (**A**), *Ficus carica* (**B**) and *Vitis vinifera* (**C**). Preparations were stained by the picro aniline blue method to visualize fungal hyphae.

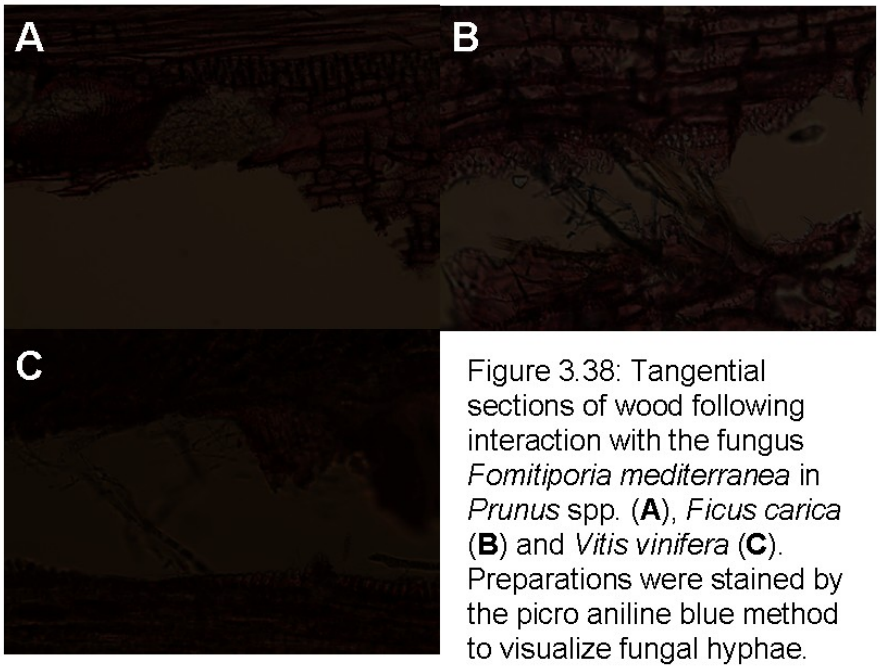


Figure 3.38: Tangential sections of wood following interaction with the fungus *Fomitiporia mediterranea* in *Prunus* spp. (**A**), *Ficus carica* (**B**) and *Vitis vinifera* (**C**). Preparations were stained by the picro aniline blue method to visualize fungal hyphae.

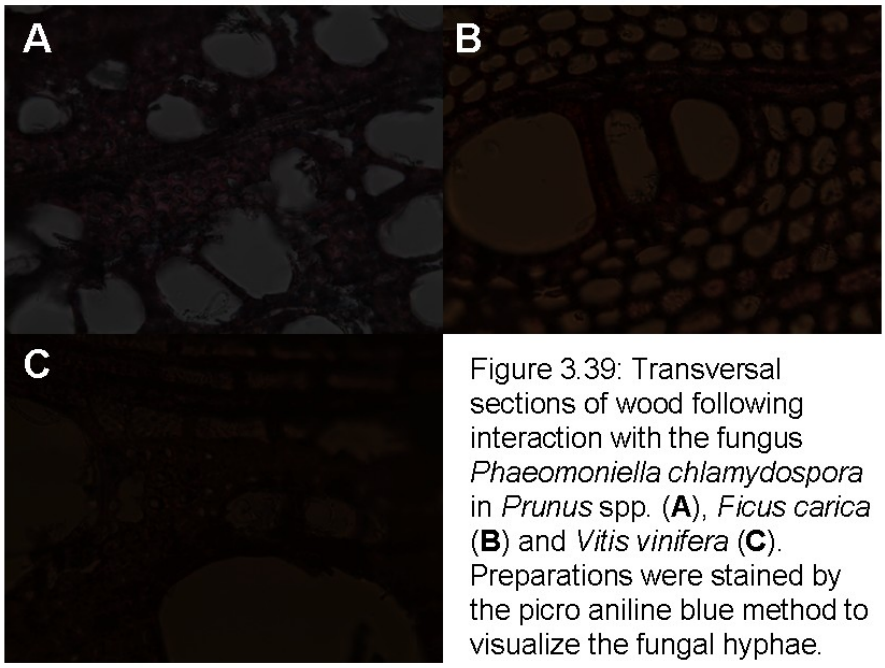


Figure 3.39: Transversal sections of wood following interaction with the fungus *Phaeomoniella chlamydospora* in *Prunus* spp. (**A**), *Ficus carica* (**B**) and *Vitis vinifera* (**C**). Preparations were stained by the picro aniline blue method to visualize the fungal hyphae.

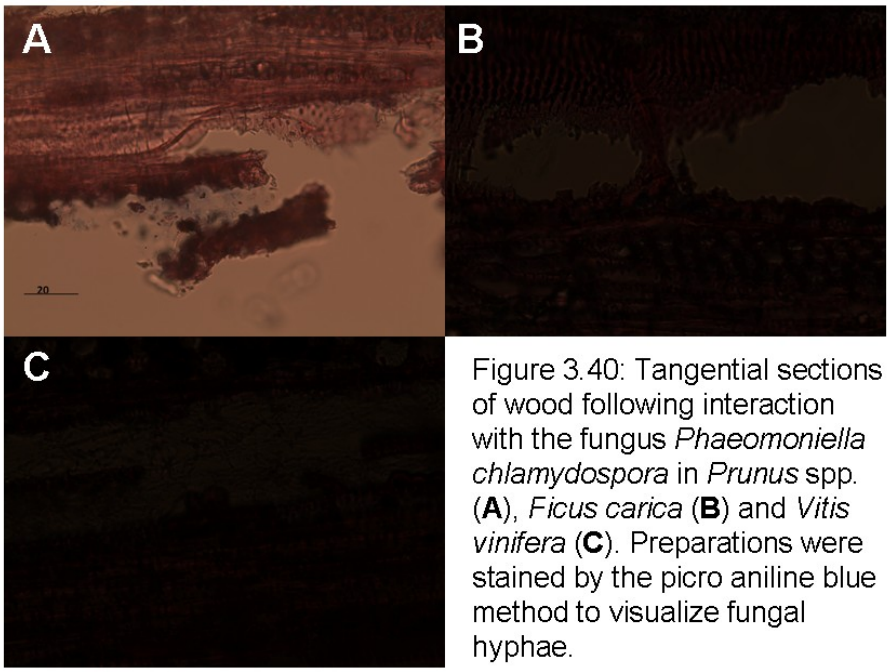


Figure 3.40: Tangential sections of wood following interaction with the fungus *Phaeomoniella chlamydospora* in *Prunus* spp. (**A**), *Ficus carica* (**B**) and *Vitis vinifera* (**C**). Preparations were stained by the picro aniline blue method to visualize fungal hyphae.

### 3.5 Test of growth in different media

The three fungal species under study (*Trametes versicolor*, *Fomitiporia mediterranea*, and *Phaeomoniella chlamydospora*) were grown in different media: PDA, agar, *Prunus* dust, *Prunus* agar, *Prunus* -, *Ficus* dust, *Ficus* agar, *Ficus* -, *Vitis* dust, *Vitis* agar, *Vitis* -. The designation of dust was used to the cases were the wood was pulverised and then used to create the media. The designation agar is used to the cases were pulverised wood was left in a incubation and the liquid fraction was mixed with agar to create the media. The designation – is used to the media created with the pulverised wood that was used in the incubation. The composition and the preparation of each medium is described in the Material and Methods section. On the images shown in Figures 3.48 to 3.58 it is possible to see the growth of the different fungi on the different media.

In what *T. versicolor* is concerned, the only difference in the fungal colony phenotype seems to occur on agar medium. The density of hyphae seems less compared with those in the other media. In *F. mediterranea*, the only difference is apparent on agar medium, as in the case of *T. versicolor*, where hyphae density seems to be lower. In the case of *P. chlamydospora* an identical difference on hyphae density on agar medium was observed. In addition, two other differences were observed in the *Prunus*, *Ficus* and *Vitis*-based media, in which the terminal white parte of the fungal colonies are wider. Another difference is one visible alteration on the colour on the surrounding area of the fungal colonies which was only visible on *Prunus* agar and *Vitis* agar media.

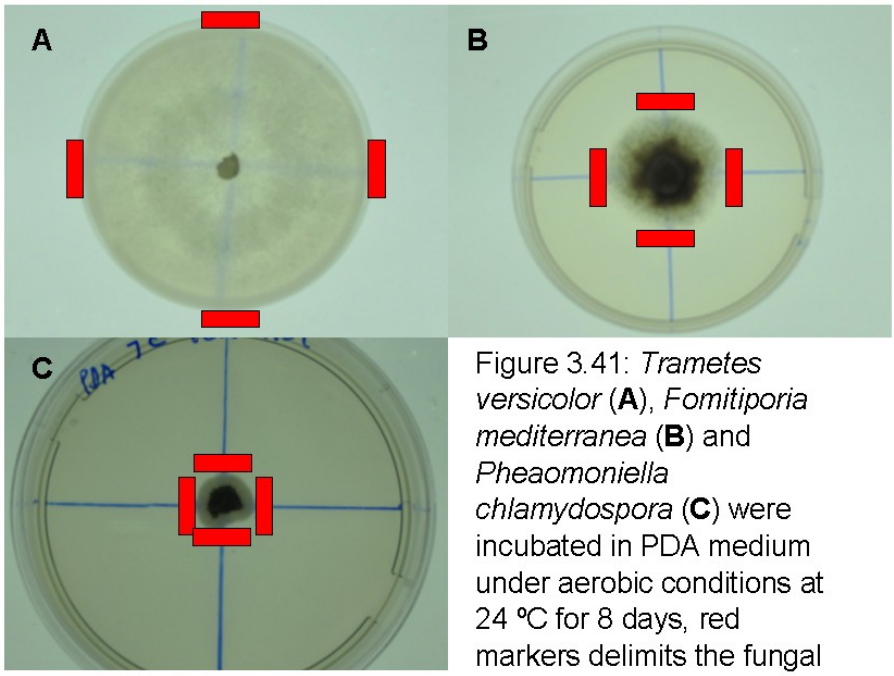


Figure 3.41: *Trametes versicolor* (**A**), *Fomitiporia mediterranea* (**B**) and *Pheaomoniella chlamydospora* (**C**) were incubated in PDA medium under aerobic conditions at 24 ºC for 8 days, red markers delimits the fungal colonies.

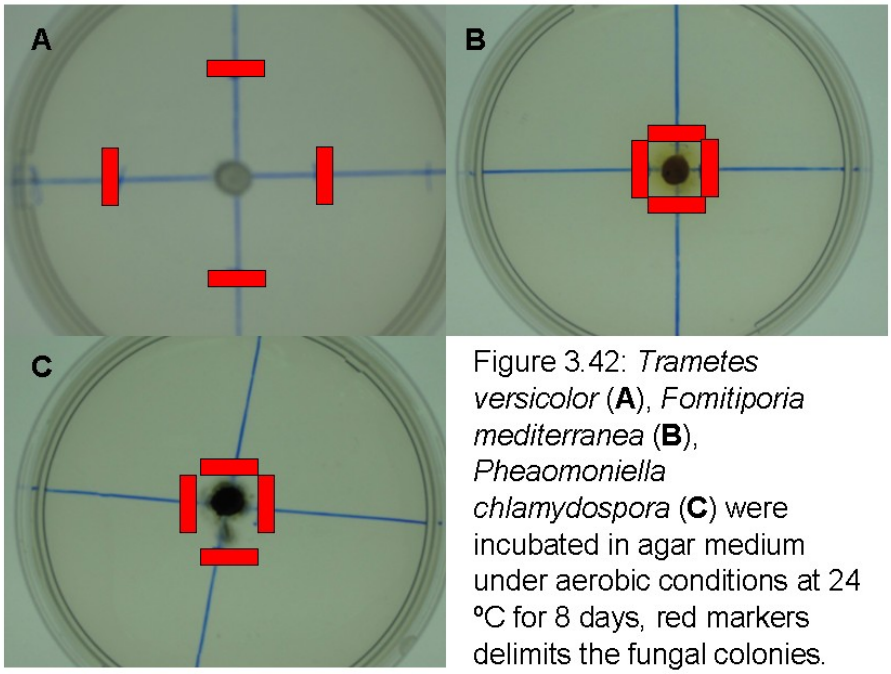


Figure 3.42: *Trametes versicolor* (**A**), *Fomitiporia mediterranea* (**B**), *Pheaomoniella chlamydospora* (**C**) were incubated in agar medium under aerobic conditions at 24 ºC for 8 days, red markers delimits the fungal colonies.

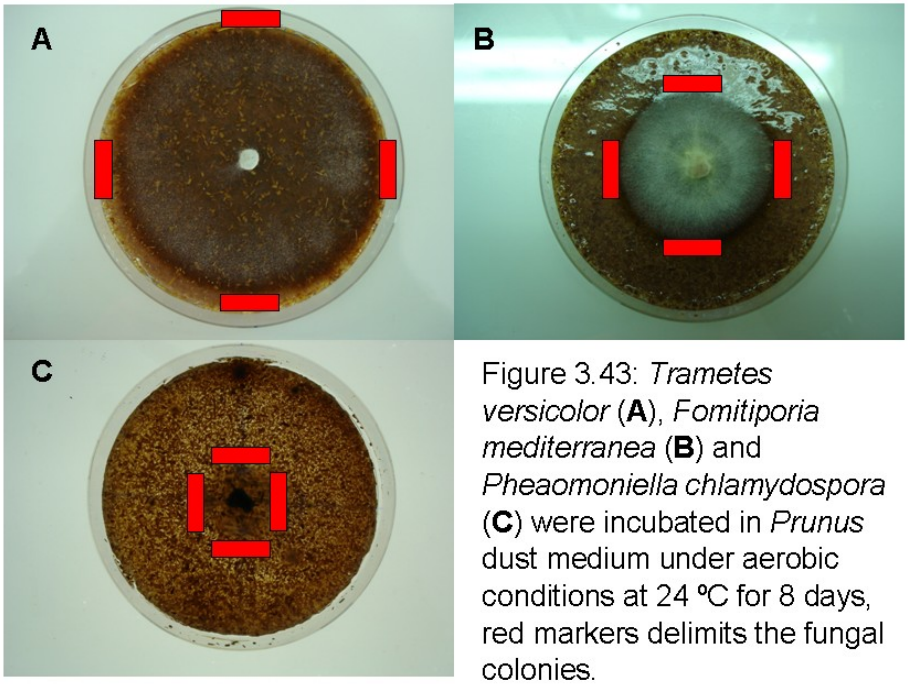


Figure 3.43: *Trametes versicolor* (**A**), *Fomitiporia mediterranea* (**B**) and *Pheaomoniella chlamydospora* (**C**) were incubated in *Prunus* dust medium under aerobic conditions at 24 ºC for 8 days, red markers delimits the fungal colonies.

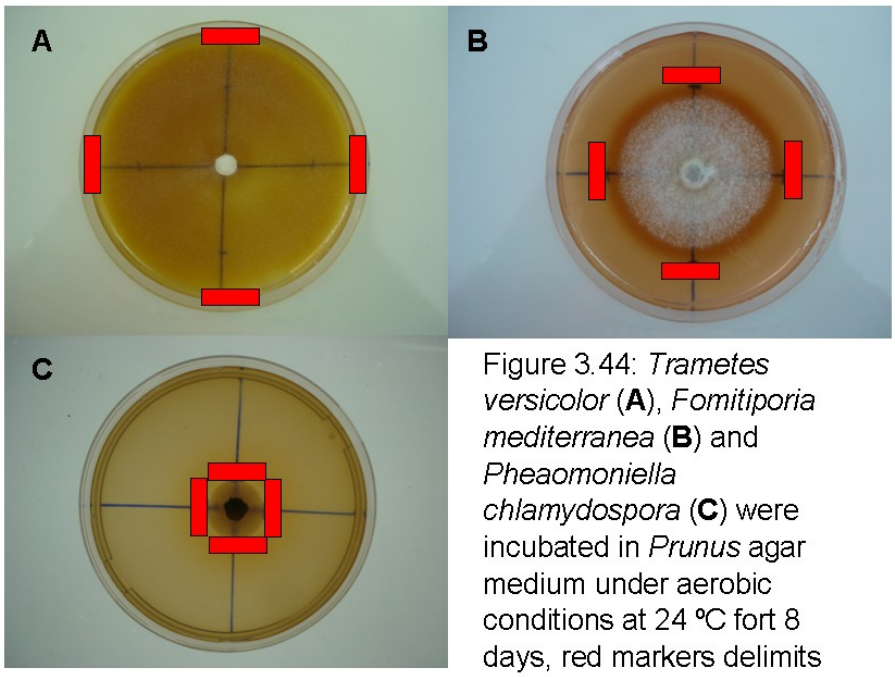


Figure 3.44: *Trametes versicolor* (**A**), *Fomitiporia mediterranea* (**B**) and *Pheaomoniella chlamydospora* (**C**) were incubated in *Prunus* agar medium under aerobic conditions at 24 ºC fort 8 days, red markers delimits the fungal colonies.

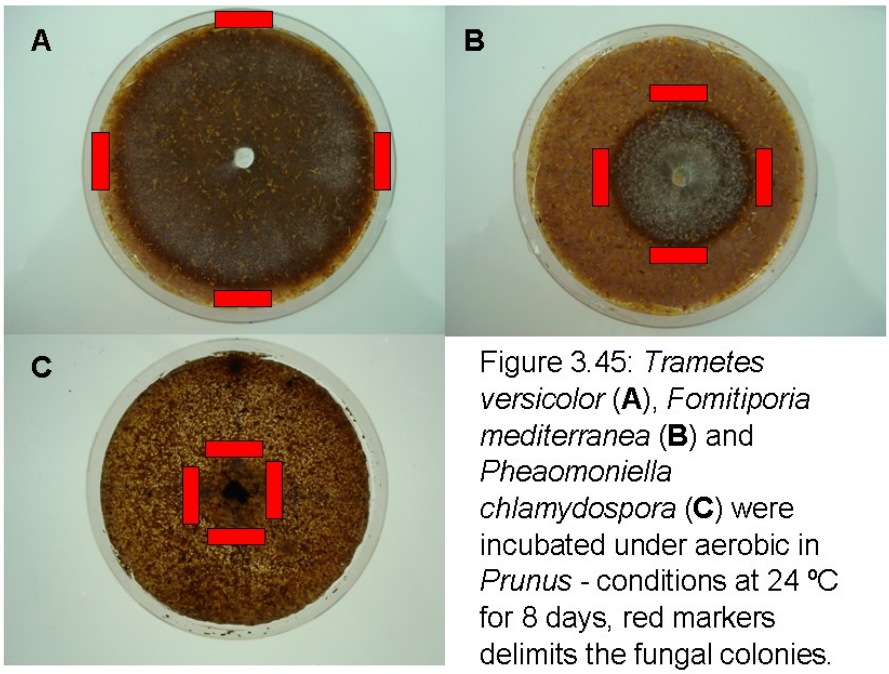


Figure 3.45: *Trametes versicolor* (**A**), *Fomitiporia mediterranea* (**B**) and *Pheaomoniella chlamydospora* (**C**) were incubated under aerobic in *Prunus* - conditions at 24 ºC for 8 days, red markers delimits the fungal colonies.

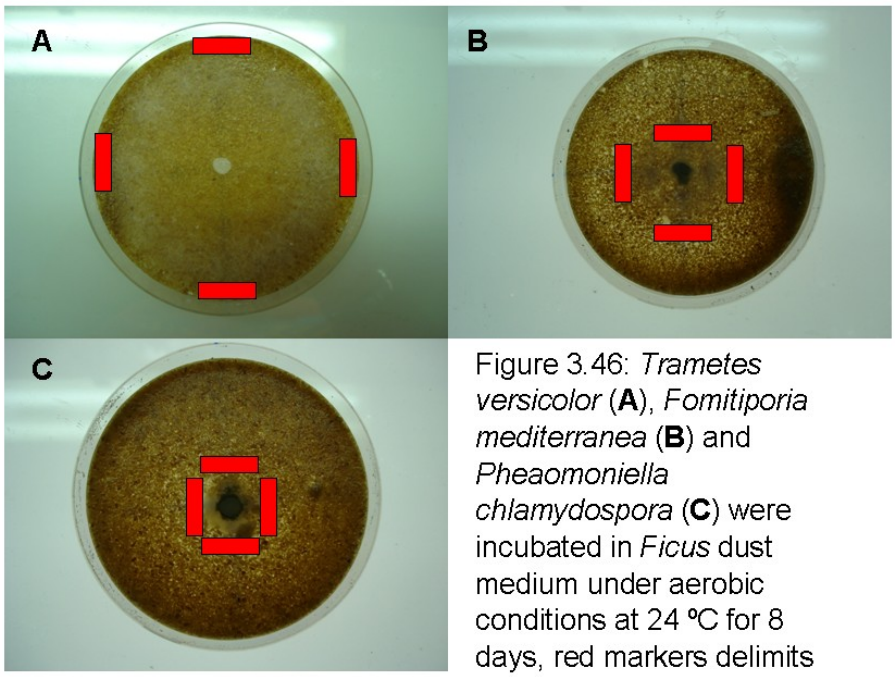


Figure 3.46: *Trametes versicolor* (**A**), *Fomitiporia mediterranea* (**B**) and *Pheaomoniella chlamydospora* (**C**) were incubated in *Ficus* dust medium under aerobic conditions at 24 ºC for 8 days, red markers delimits the fungal colonies.

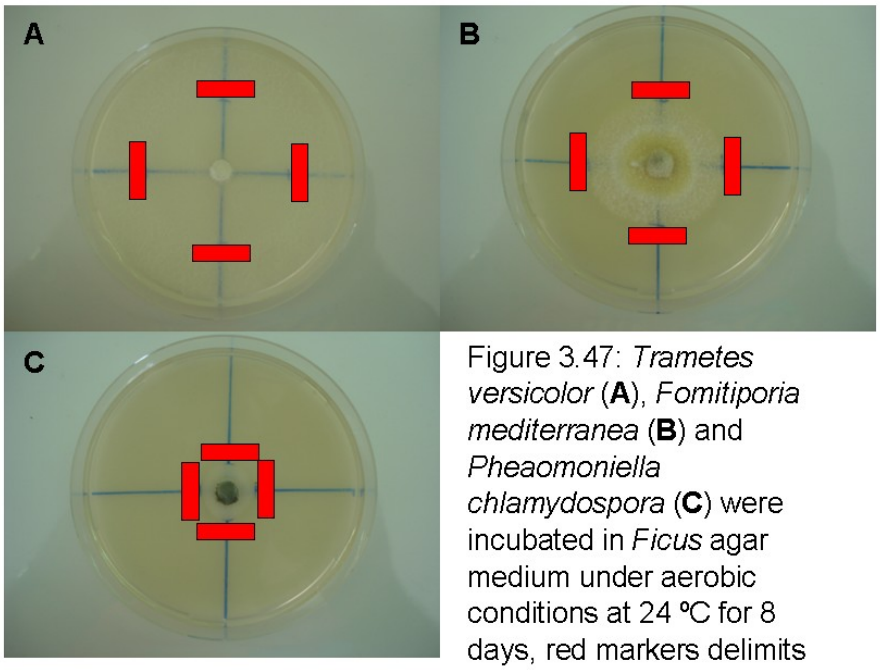


Figure 3.47: *Trametes versicolor* (**A**), *Fomitiporia mediterranea* (**B**) and *Pheaomoniella chlamydospora* (**C**) were incubated in *Ficus* agar medium under aerobic conditions at 24 ºC for 8 days, red markers delimits the fungal colonies.

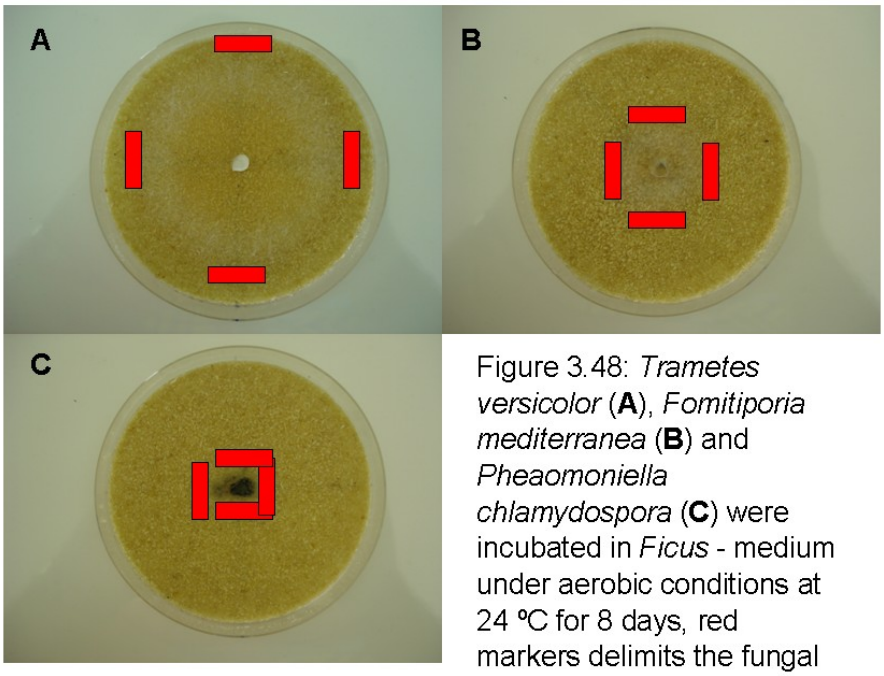


Figure 3.48: *Trametes versicolor* (**A**), *Fomitiporia mediterranea* (**B**) and *Pheaomoniella chlamydospora* (**C**) were incubated in *Ficus* - medium under aerobic conditions at 24 ºC for 8 days, red markers delimits the fungal colonies.

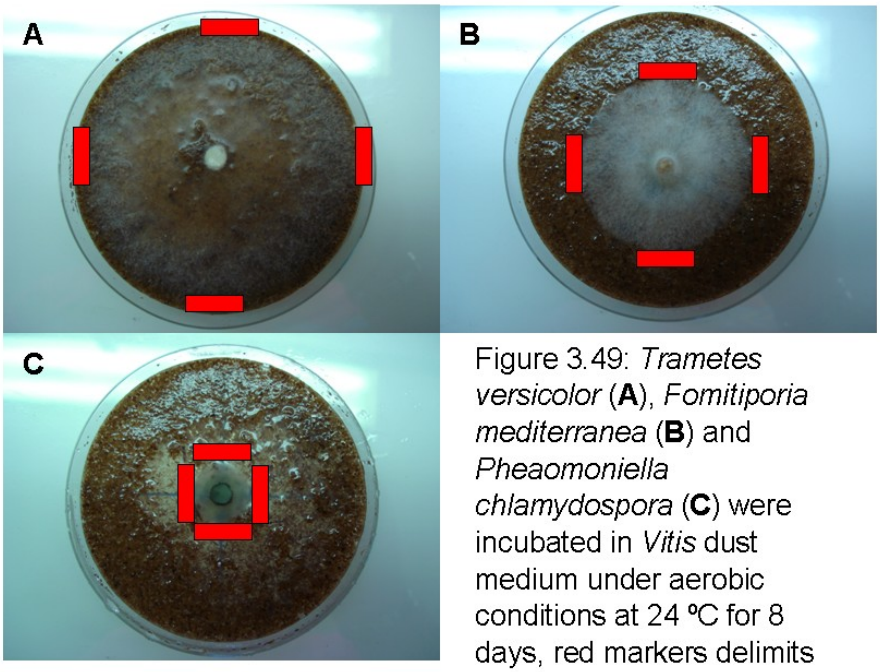


Figure 3.49: *Trametes versicolor* (**A**), *Fomitiporia mediterranea* (**B**) and *Pheaomoniella chlamydospora* (**C**) were incubated in *Vitis* dust medium under aerobic conditions at 24 ºC for 8 days, red markers delimits the fungal colonies.

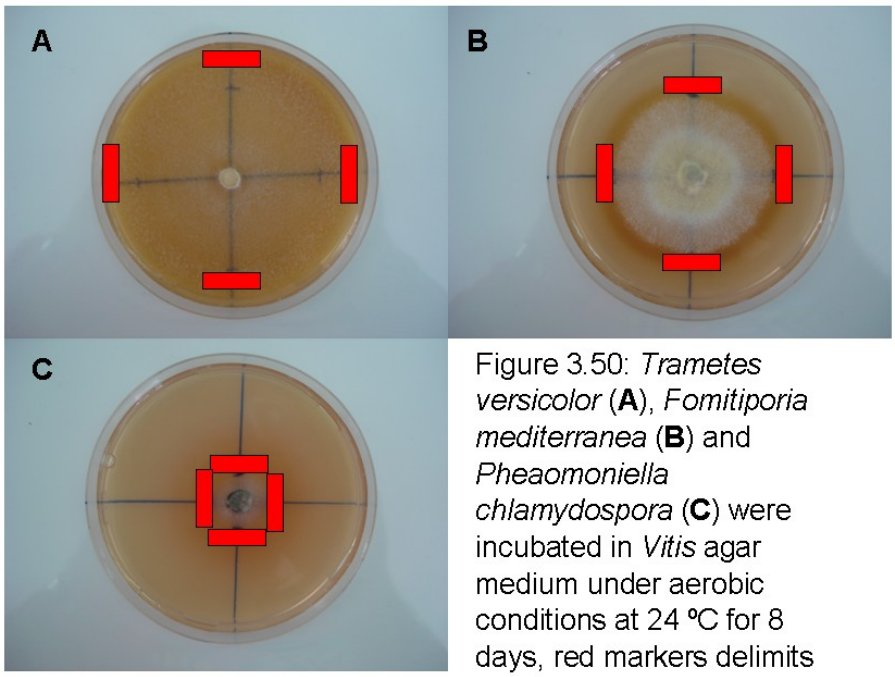


Figure 3.50: *Trametes versicolor* (**A**), *Fomitiporia mediterranea* (**B**) and *Pheaomoniella chlamydospora* (**C**) were incubated in *Vitis* agar medium under aerobic conditions at 24 ºC for 8 days, red markers delimits the fungal colonies.

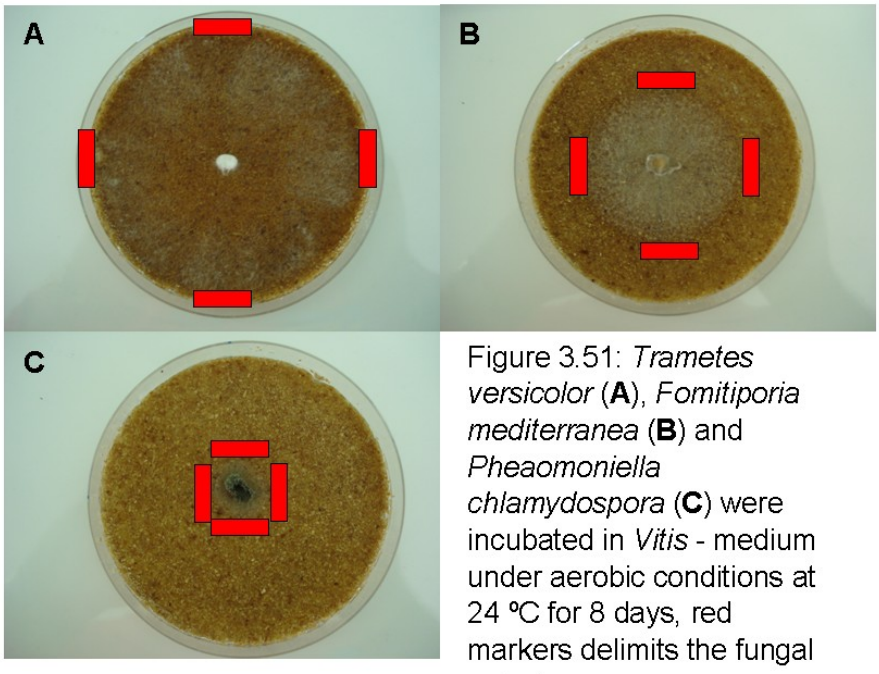


Figure 3.51: *Trametes versicolor* (**A**), *Fomitiporia mediterranea* (**B**) and *Pheaomoniella chlamydospora* (**C**) were incubated in *Vitis* - medium under aerobic conditions at 24 ºC for 8 days, red markers delimits the fungal colonies.

On the graphics displayed in figures 3.59 to 3.64 it is possible to see the rate of growth of the fungi under study in the different media and at different times (4 and 8 days). Comparisons were made among the different media of the same wood substrate. The differences that are significant (p-value<0.05 and p-value≥0.01) are marked with *, the differences highly significant (p-value<0.01) are marked with **, and the differences not significant (p-value >0.05) are unmarked.

In the case of *T. versicolor* at 4 days, only the fungus grown in *Ficus* dust and *Ficus* – have not show significant differences. The other cases have all differences highly significant. At the 8$^{th}$ day of growth, the significant differences were only observed between *Ficus* dust and *Ficus* -.

At the 4$^{th}$ day of growth with *F. mediterranea,* the only highly significant difference was observed in *Vitis* dust, and *Vitis* agar and *Vitis* -. Other significant differences are between *Prunus* agar and *Prunus* dust, and also in *Ficus* dust which exhibits a significant difference with *Ficus* agar and *Ficus* -. At the 8$^{th}$ day, all fungi which grew on agar media had reached the end of the plate and so was not further considered. The fungus that grew on *Prunus* agar showed a significant difference with *Prunus* - and a highly significant difference with *Prunus* dust. However, *Prunus* dust and *Prunus* – do not show a significant difference between themselves. In the fungi that grew on *Ficus* dust, the difference is high compared to *Ficus* agar and *Ficus* -. *Vitis* agar exhibits a significant difference when compared to *Vitis* -, with both sharing a highly significant difference with *Vitis* dust.

At the 4$^{th}$ day, the only difference observed in *P. chlamydospora* was in *Prunus dust*, were the fungus underwent a slower growth. At the 8$^{th}$ day this difference disappeared, but other become apparent: *Prunus* - produced a highly significant difference when compared to *Prunus* dust and *Prunus* agar, *Ficus* agar with *Ficus* -, and finally *Vitis* agar produced a significant difference with *Vitis* -.

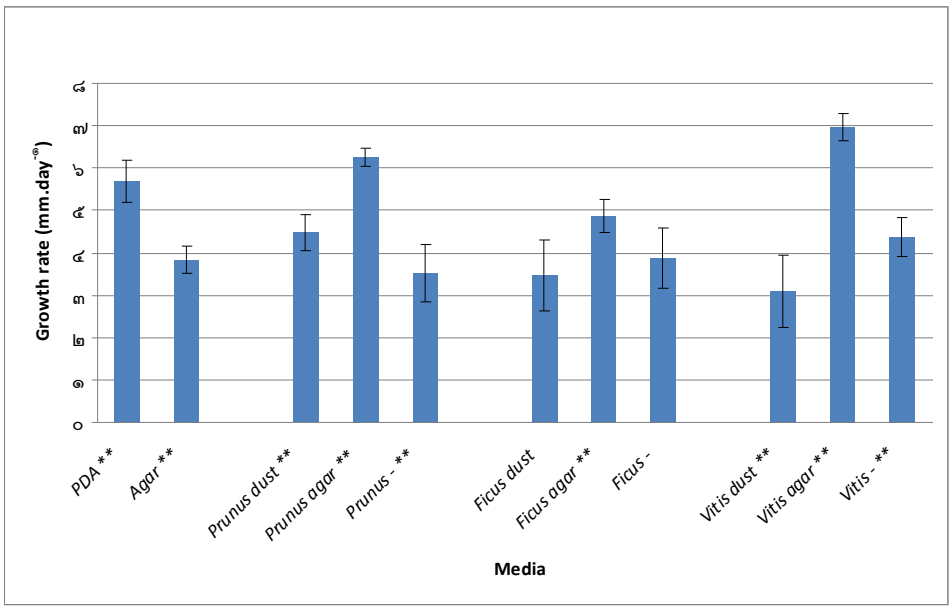


Figure 3.52: Growth rate expressed as the rate of increase of the colony diameter (mm $day^{-1}$) of *Trametes versicolor*, at 24 ºC, after 4 days, under aerobic conditions, showing the respective standard deviation magnitude (n=12).

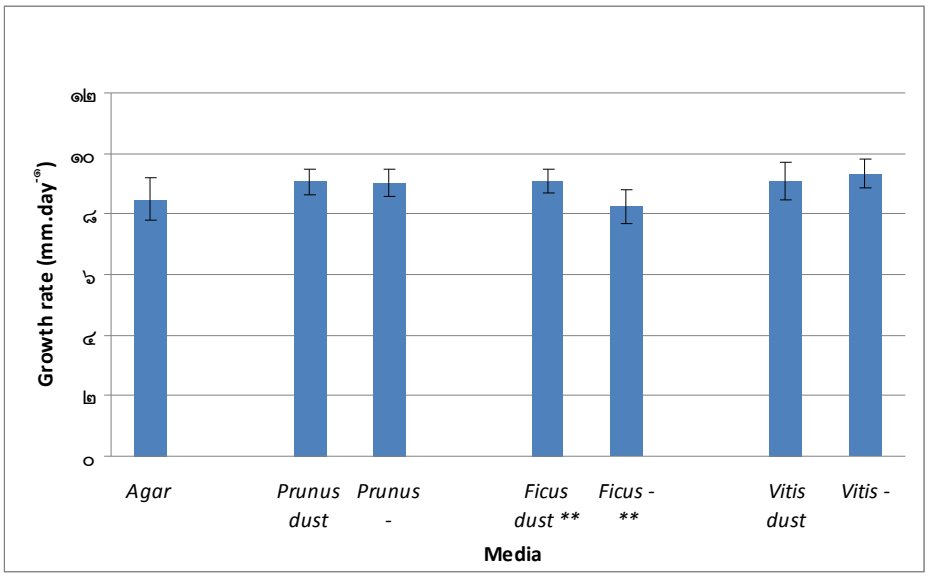


Figure 3.53: Growth rate expressed as the rate of increase of the colony diameter (mm $day^{-1}$) of *Trametes versicolor*, at 24 ºC, after 8 days, under aerobic conditions, showing the respective standard deviation magnitude (n=12).

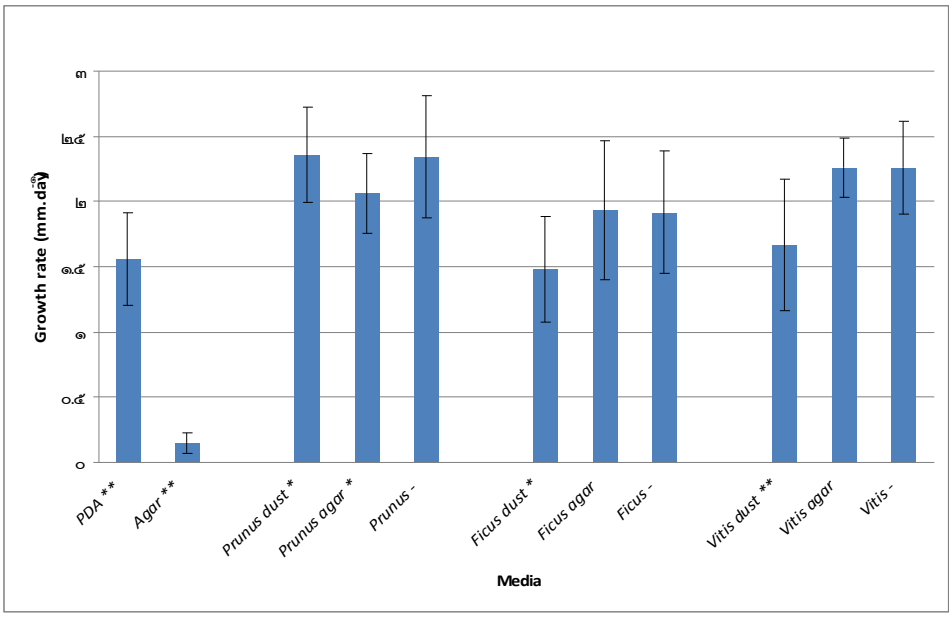


Figure 3.54: Growth rate expressed as the rate of increase of the colony diameter (mm day$^{-1}$) of *Fomitiporia mediterranea*, at 24 ºC, after 4 days, under aerobic conditions, showing the respective standard deviation magnitude (n=12).

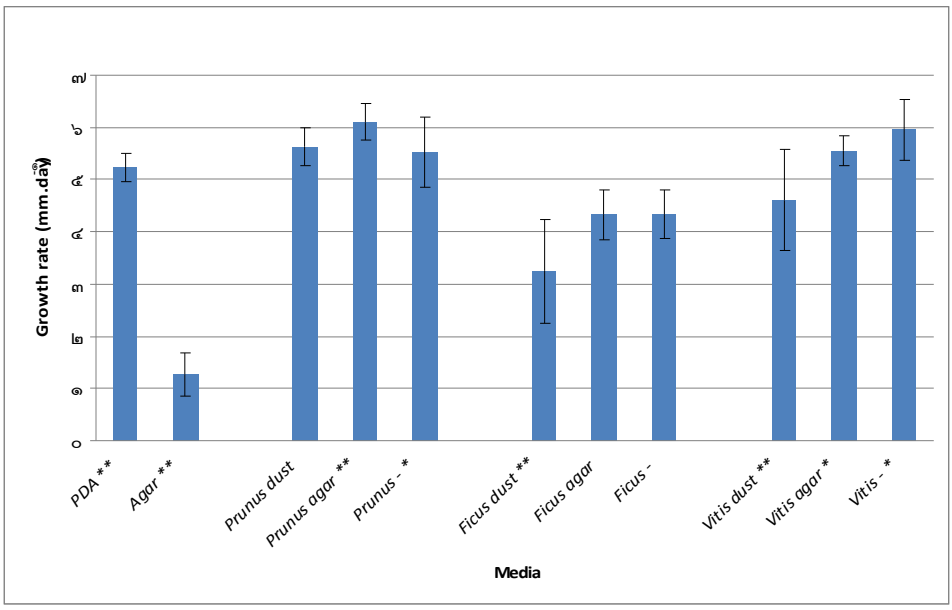


Figure 3.55: Growth rate expressed as the rate of increase of the colony diameter (mm day$^{-1}$) of *Fomitiporia mediterranea*, at 24 ºC, after 8 days, under aerobic conditions, showing the respective standard deviation magnitude (n=12).

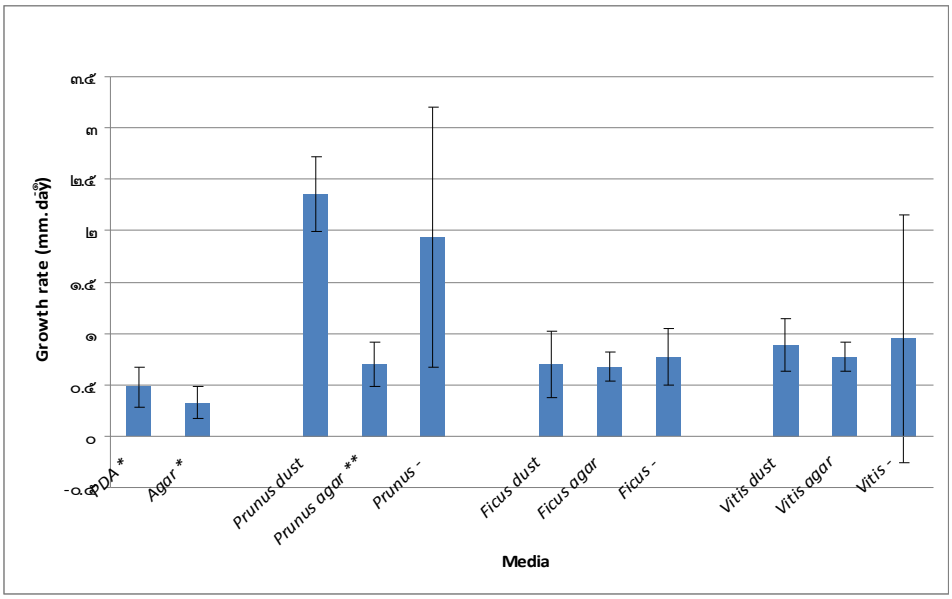


Figure 3.56: Growth rate expressed as the rate of increase of the colony diameter (mm day$^{-1}$) of *Pheaomoniella chlamydospora*, at 24 ºC, after 4 days, under aerobic conditions, showing the respective standard deviation magnitude (n=12).

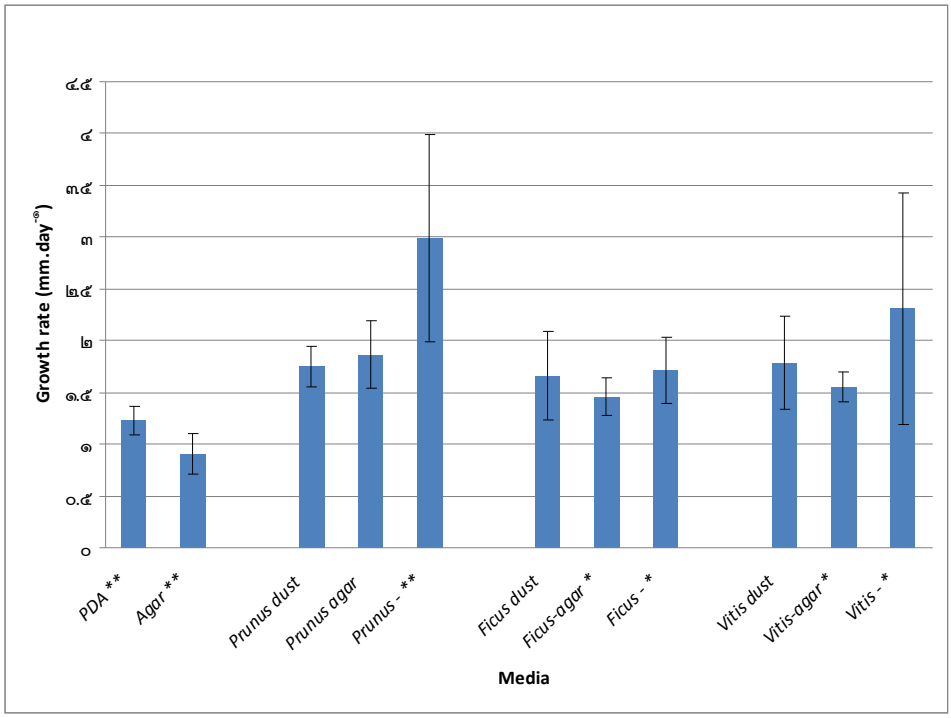


Figure 3.57: Growth rate expressed as the rate of increase of the colony diameter (mm day$^{-1}$) of *Pheaomoniella chlamydospora*, at 24 ºC, after 8 days, under aerobic conditions, showing the respective standard deviation magnitude (n=12).

### 3.6 Test of peripheral growth unit

This test was made to understand the differences of the growing hyphae of the fungi under study (*Trametes versicolor*, *Fomitiporia mediterranea*, and *Phaeomoniella chlamydospora*) on the different media. Hyphae of *T. versicolor* were grown under different conditions for 48 h, whereas *F. mediterranea* and *P. chlamydospora* were grown during 72 h. The conditions were the same as those described in section **3.5**, using the following media: PDA, agar, *Prunus* dust, *Prunus* agar, *Prunus* -, *Ficus* dust, *Ficus* agar, *Ficus* -, *Vitis* dust, *Vitis* agar, and *Vitis* -. The explanations for the designations for the terms dust, agar and – in the media are give in the Material and Methods section. The terminal parts of the hyphae were measured until at least the second ramification. The results obtained were subsquently divided by the number of segments measured. For each case, 45 hyphae were measured. No differences were detected at the level of the phenotype, the structure and shape of the hyphae have no obvious differences (except in what concern to differences between species).

The graphics with the average and standard deviation of Peripheral Growth Unit (PGU) are illustrated on Figures 3.76 to 3.78. The fungi have grown during 48 h (*T. versicolor*) or 72 h (*F. mediterranea* and *P.chlamydospora*). The differences that are significant (p-value<0.05 and p-value≥0.01) are marked with *, the differences highly significant (p-value<0.01) are marked with **, and the differences not significant (p-value >0.05) are unmarked.

On *T. versicolor,* the PGU show highly significant differences between *Ficus* dust and *Ficus* – media. Other highly significant difference was detected between in *Vitis* – and both *Vitis* dust and *Vitis* agar.

In the PGU of *F. mediterranea,* highly significant differences were detected in *Prunus* agar when compared with the other media of *Prunus,* and in *Vitis* dust when compared with the other media of *Vitis*.

In the PGU of *P. chlamydospora,* highly significant differences were detected in all media of *Prunus*. No other differences were observed.

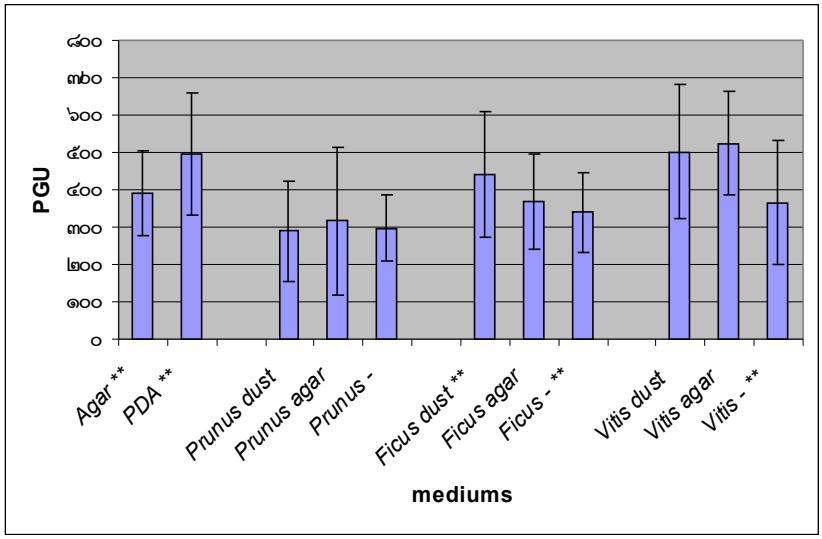


Figure 3.58: Avarage of the peripheral growth unit (measured in µm) of *Trametes versicolor*, at 24 ºC, after 48 h, under aerobic conditions. The corresponding standard deviation bars are indicated (n=45).

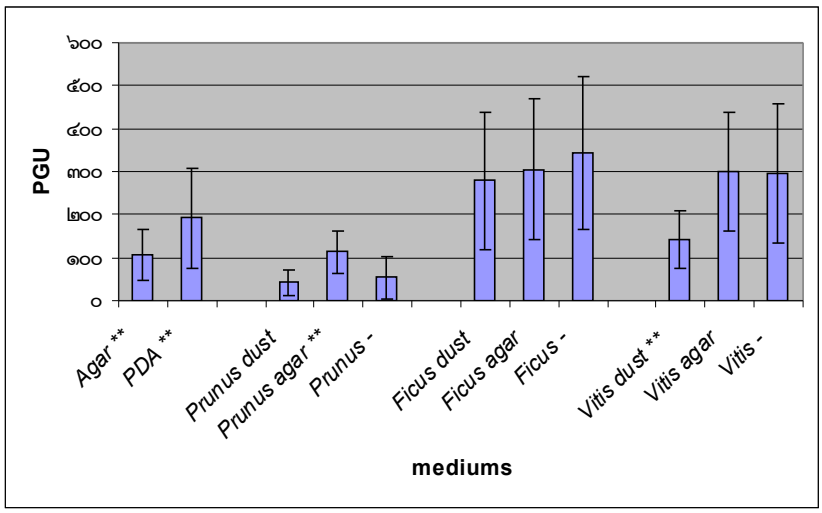


Figure 3.59: Avarage of the peripheral growth unit (measured in µm) of *Fomitiporia meditrranea*, at 24 ºC, after 72 h, under aerobic conditions. The corresponding standard deviation bars are indicated (n=45).

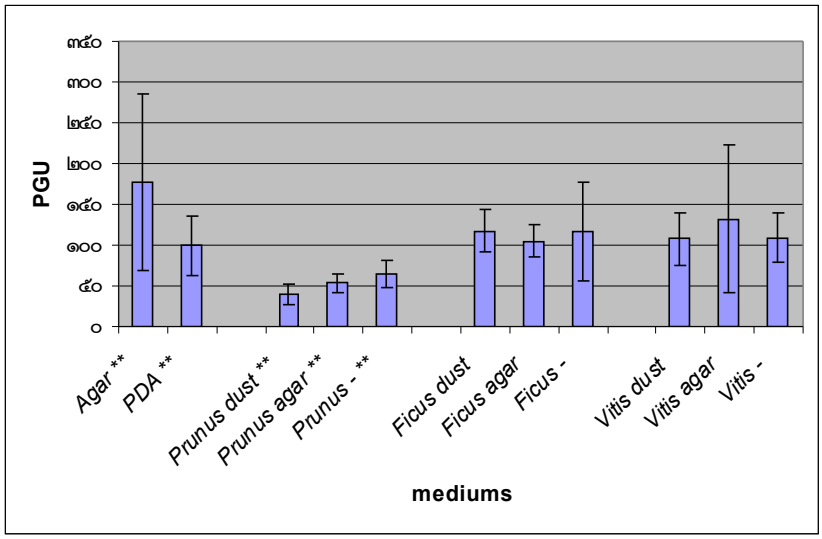


Figure 3.60: Avarage of the peripheral growth unit (measured in µm) of *Pheaomoniella chlamydospora*, at 24 ºC, after 72 h, under aerobic conditions. The corresponding standard deviation bars are indicated (n=45).

# 4. DISCUSSION

Among the main objectives that this study set out to achieve was the understanding of the way in which *Phaeomoniella chlamydospora* and *Fomitiporia mediterranea* interact with their woody substrate. Initially it was decided to investigate oxygen relationships for the test fungi, whilst at a later stage the focus shifted towards interspecific interactions and wood colonisation abilities of each fungus.

It was previously unknown whether these fungi could grow under anaerobic conditions. The results presented in this thesis clearly demonstrate that the fungi do not grow in the absence of oxygen, and resume growth if subsequently exposed to aerobic conditions, which makes them aerobic facultative fungi. However, as no studies were carried out to determine the capacity of these fungi to grow under microaerophilic conditions, it remains unclear whether these fungi are capable of growth under such conditions. As the *in vivo* oxygen status inside the esca infection and diseased grapevine wood is unknown, it is possible to speculate that *P. chlamydospora* might invade the plant because it can grow under microaerophilic conditions. In many fungal species oxygen is necessary for spore germination and hyphal growth, as the activity of mitochondria is essential in these processes (Subíková and Subík, 1974). Spore germination is complex at the metabolic level (Sussman and Halvorson, 1966) and requires energetic support (Allen, 1965). This evidence supports the view that the woody tissue may not be an anoxic environment and that *P. chlamydospora* may be able to continue growth in the presence of reduced amounts of oxygen. Nevertheless, ad hoc studies will need to be performed in the future to address this issue and to provide some conclusive evidence on this respect. For all these reasons it is possible to conclude that at least some oxygen must exist in the grapevine wood for the esca-related fungi to develop and grow.

It has been suggested that *P. chlamydospora* is the first fungus to invade grapevine in the esca infection and disease because it has the capacity to suppress the defences of *Vitis vinifera*, which in turn becomes more susceptible to colonisation by *Phaeocremonium aleophilum* and by opportunist fungi (Valtaud, 2009). It is known that some esca fungi produce chemical compounds that are toxic to other fungi and grapevine. However in the interaction of the esca fungi the toxic compounds produced are converted into other chemicals, only toxic to plants (Glaser *et al*., 2009). To explain this, it would be interesting to know the susceptibility of these other fungi to grapevine defence mechanisms. It is possible that these fungi may be unable to overcome the defences of the vine, but they may overcome the toxicity of *P. chlamydospora* metabolites. This hypothesis is partly supported by the results presented in this study. When *Trametes versicolor*, which is not a fungus

associated with esca, interacts with *P. chlamydospora*, it causes the accumulation of hydrogen peroxide and superoxide ion. Furthermore, even when ROS were not detected, an alteration in the phenotype of *T. versicolor* was observed, which was associated with a slower growth when compared to the controls. During the interaction between the esca fungi *P. chlamydospora* and *F. mediterranea*, no accumulation of ROS was evident, indicating a possible compatible interaction between the two fungi. It is improbable that the accumulation of ROS possibly produced upon the interaction between *T. versicolor* and *P. chlamydospora* may be linked solely to the direct interaction between the fungi as ROS are normally only present in the interaction zone (Silar, 2005). The large accumulation of ROS in *T. versicolor* is probably one oxidative stress caused by other chemicals produced by *P. chlamydospora* and may be the generalised response of the former fungus to a non-compatible interaction. This toxicapacity of *P. chlamydospora* may be one strategy of survival of the fungus compensating for its slow rate of growth. In the first instance, the toxic metabolites produced by the fungus make it more competitive during interspecific interactions. It is known that some fungi produce mycotoxins during the interactions, but not always these mycotoxins lead to an advantage to the producer fungus; in many cases, it is not obvious the advantages taken (Xu *et al*., 2007). The oxidative stress observed in *T. versicolor* may be one response to *P. chlamydospora* to protect its substrate, but without obvious advantages, because although *T. versicolor* seems affected, it continues its development.

In the present study, *P. chlamydospora* has been observed to cause some degradation in the wood species tested. This degradation was not limited to grapevine, but extended to other wood species that are not generally associated with the occurrence of the fungus. Microscopy analyses confirmed the occurrence of damage to the wood, which affected lignin especially in the cell wall of xylem vessels. This phenomenon was greatly accentuated in the case of *F. carica*. When cellulose was investigated, some degradation was also detected, which was manifested by a light blue coloration, indicating low levels of cellulose. These results are in disagreement with the work of Valtaud (Valtaud *et al*., 2009), which concluded that *P. chlamydospora* does not have the ability of degrade wood. It is important to take into account that *P. chlamydospora* displays, as discussed before, a rate of growth which is very slow when compared to other wood colonising fungi and so the degradation of wood will take longer. It must also be noted that Valtaud and colleagues (2009) performed their work with living plants, whereas the present study was undertaken in sterilized wood bocks, thus creating different conditions and so producing different results. *P. chlamydospora* probably acts producing and releasing toxins like phenolic compounds and phytotoxic pullulans (Bruno and Sparapano, 2000). Under sterilizing wood conditions, such toxins may be irrelevant, as physiological responses by the plant and/or

microorganisms are not expected to occur - maybe under such conditions the ability to of *P. chlamydospora* to degrade wood is most apparent.

In the present study, the wood degrading abilities of *F. mediterranea* were also evaluated. Particularly, it was evident that this fungus could degrade the wood of *V. vinifera* without prior colonisation by *P. chlamydospora*. It is worth of note, however, that technical difficulties were encountered in the preparation of fresh and sterile wood samples for the degradation tests, as these became contaminated during the fungi exposure test. Thus, tests were conducted on dried wood samples that were prepared and sterilised by radiation. For this reason, the objective of the study was modified slightly and focused not on colonisation of green wood by esca inhabiting fungi, but rather on the wood degradation capabilities of these fungi. It seems therefore irrelevant that there was no interaction between the two esca-associated fungi within the wood substrate. Two further points need consideration. The first is that several compounds in the wood may be affected and suffer some degradation processes during and/or following the radiation treatment, including antifungal compounds. But in another assay, the three types of non sterilized wood blocks were subjected to penetration by the three fungal species and *F. mediterranea* succeeded to penetrate in the wood of grapevine (data not show). The work of Fischer (2008) may explain the situation, since he discovered that the spores of *F. mediterranea* can germinate in grapevine without the support of *P. chlamydospora*. This observation may clarify why *F. mediterranea* hyphae are able to penetrate the three fresh species of wood.

In the tests of growth in different media, fungi were put to grown in different media conditions. The media used was:

1 PDA.

2 Agar.

3 *Prunus* dust, *Ficus* dust and *Vitis* dust, that consisted in agar mixed with dust of the respective wood) (see Materials and Methods section).

4 *Prunus* agar, *Ficus* agar and *Vitis* agar, that consisted on a broth of the wood mixed with agar (see Materials and Methods section).

5 *Prunus* -, *Ficus* - and *Vitis* –, that consisted on wood dust used to create the broths mixed with agar (see Materials and Methods section).

In the media where the broth of wood was used, the soluble compounds become immediately available to fungi. This case can explain in some cases the faster growth of *T. versicolor* in these media. However the other fungi do not have this behaviour. Some compounds are not soluble and are only available in the wood dust. The dust that was used to generate the broth would have less soluble nutrients then the wood that was directly mixed with agar. The insoluble compounds can be digested and then absorb by the fungi, in this case the fungi will have more nutrients but will need more time for they become

available. However this was not the case, the only case where the fungi grown faster in wood dust was in *Prunus* dust (both in *F. mediterranea* and *P. chlamydospora*) and only at the 4th day, in the 8th day this result was no more observed. In other case it would be expected that the wood dust used to create the broth ad less soluble nutrients and so the media that were created with them, would have the lowest grown, but this is not so simple, in many cases the fungi have one equal grown ore even faster when compared with other media.

On the test of growth other thing to have in consideration is the presence or absence of anti-fungal compounds. Fungal compounds can be soluble or insoluble in water (it can append that both type of anti-fungal compounds coexists in the wood). They can also have more effect or even be specific to some species of fungi, but fungi can also have mechanisms that suppress this plant defence. It this possible that the wood of *Ficus carica* have some anti-fungal compounds, the fungi *T. versicolor* and *F. mediterranea* in general ad grown more slowly on these media, when compared with other media. Other reason for this situation can be caused by some type of less affinity of the fungi to the type of wood. In *P. chlamydospora* this situation in *Ficus* media as not observed, this can be caused the rate of growth very low of this fungus, it can append that this specie need of more time to be observed some difference. Anti-fungal compounds can also explain why *F. mediterranea* and *P.chlamydospora* in some cases grown more in the media of *Prunus* – and *Vitis* -. It is possible that both types of anti-fungal compounds (soluble and insoluble) are present in the wood, the wood that was not used to create the broth have the two types of anti-fungal compounds that caused some difficulty on the development of the fungi. The media that were created with the broth ore the wood used on the broth have only one type of anti-fungal compounds and fungi grow more easily on those cases. However it is on very difficult to explain the results, many of the hypotheses discussed here are only speculation. There is no test that confirms the presence or absence of anti-fungal compounds. The availability of the nutrients that are in the wood passed to the media, or not, is also speculation. There is no guaranty that in the media of wood dust directly mixed with agar, the soluble matter do not passed to the agar, in fact that is very unlikely. Also it is improbably that the wood used to create the broths passed all the soluble matter (nutrients and anti-fungal compounds) to the water, some substances stay on the wood, and become available in the media. For now the explanations given here are only hypothesis.

The tests of wood degradation did not produce results in agreement with those from the growth in different media. In the wood degradation experiments, the wood of *Prunus* spp. was the one that suffered less degradation for all fungal species tested. The degradation observed for the other woods (*F. carica* and *V. vinifera*) was intense, but no significant differences were detected between them. It is difficult to explain how the wood of *Prunus*

was the less degraded, when the fungi did not demonstrate any apparent difficulty to grow on such media. At the same time, the fungi demonstrated a slower growth in the *Ficus* media, but the wood of *F. carica* suffered more intense degradation when compared to the wood of *Prunus* and equal degradation when compared to the wood of grapevine.

No morphological differences were seen in the test of hyphal growth. The only differences observed were between species of fungi. Therefore, it seems that the media used in this work do not cause any morphological differences in the fungal hyphae, except in the PGU. *F. mediterranea* and *P. chlamydospora* seem to display a major PGU in the wood of *Ficus* and grapevine than in *Prunus*. However, these results are inconclusive and it is for now impossible to make one conclusion or make one comparison with the test of growth in different mediums.

Esca is certainly a very complex disease, probably involving intricate interactions among the several contributing fungal species. The precise sequence or simultaneous infection of grapevine wood by the fungi depends not only on the interactions among themselves but also on the environmental conditions, i.e. the vine wood. As a whole, the work presented in this thesis aimed at answering a few simple questions on complex issues such as, for example, the basic knowledge on the fungi oxygen requirements for vine wood colonization.

# 5. FUTURE PERSPECTIVES

It will be interesting to study the compounds present in agar in confrontation zones of fungi. It was proving in others studies that not only ROS are present in the interactions of fungi. The compounds can have antifungal properties, can be toxic to grapevine, or even had other properties unknown, in some cases some fungi can be stimulated, in others can be inhibited. The enzymatic activity of fungi in interaction must be study to; the enzymes of several fungi can cooperate in degradation of wood. These interactions must be study to, on wood and see how wood is degraded and see if there is any difference like more degradation or if fungi enter in competition and attack each other. Finally in the case of interactions, the interaction between *Phaeomoniella chlamydospora* and *Trametes versicolor*, this last fungus had suffered some kind of stress caused by the other. Did this was only one particular case, or did *P. chlamydospora* have the ability of cause several stress in some competitors.

Other interesting experiment is the test of rate of growth in conditions of low oxygen. It would be interesting to know if esca fungi are able to growth in conditions of low oxygen, or not. Of course if is proved that esca fungi is able to grow in low oxygen, it must be investigated the levels of $O_2$ on wood and seem if it as some importance in infection.

Finally it could be interesting study the wood or chemicals presents in fig. the wood of fig suffer intense degradation, but by other side, the fungi do not grow so well in mediums of fig. Why the wood of fig is intensely degraded but fungi development is minor compared with development in other mediums?

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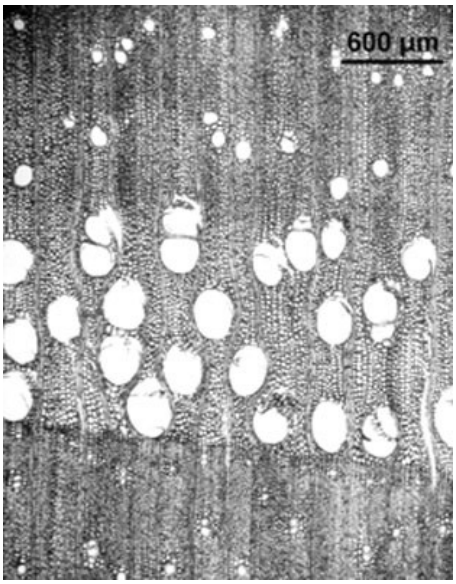
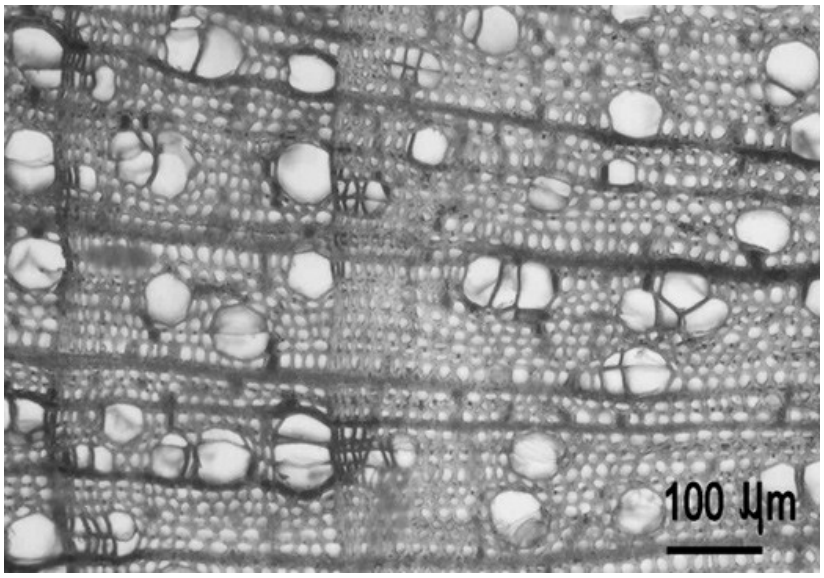
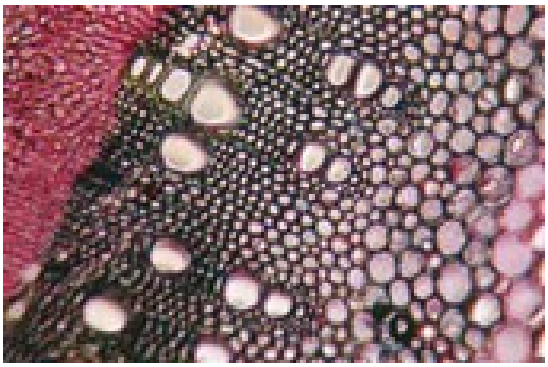
# ANNEXES

# ANNEX A:

Table 7.1: Summary table of wood density for selected wood species

| Specie | Density Kg $m^{-3}$ | Image / Notes |
|---|---|---|
| *Vitis vinifera* | 547.37 |  Trunk of grapevine infected with esca http://www.biorede.pt/ http://www.worldagroforestrycentre.org |
| *Fagus sylvatica* | 650 |   http://www.scielo.cl/fbpe/img/maderas/v8n3/fig6_art07.jpg http://www.worldagroforestrycentre.org |
| *Fagus* | 620 | Image not found |

| | | |
|---|---|---|
| *cunnighamii* | | http://www.worldagroforestrycentre.org |
| *Fagus grandifolia* | 660 |  <br>http://upload.wikimedia.org/wikipedia/commons/thumb/0/0b/Taxus_wood.jpg/250px-Taxus_wood.jpg<br>http://www.worldagroforestrycentre.org |
| *Fagus longipetiola* | 600 / 700 | Image not found<br>http://www.worldagroforestrycentre.org |
| *Betula alleghaniensis* | 620 | Image not found<br>http://www.worldagroforestrycentre.org |
| *Betula pendula* | 657 |  <br>http://www.scielo.cl/fbpe/img/gbot/v65n1/fig1-4.jpg<br>http://www.worldagroforestrycentre.org |
| *Betula* | 600 / 719 | Image not found |

| | | |
|---|---|---|
| *pubescens* | | http://www.worldagroforestrycentre.org |
| *Quercus petraea* | 673 / 720 | Image not found<br>http://www.worldagroforestrycentre.org |
| *Quercus robur* | 689 / 720 | Image not found<br>http://www.worldagroforestrycentre.org |
| *Ficus carica* | 500 / 600 |  <br>http://www.fotosearch.com/photos-images/microphotography.html<br>http://www.worldagroforestrycentre.org |
| *Prunus serotina* | 540 | Image not found<br>http://www.worldagroforestrycentre.org |
| *Prunus arborea* | 435-530 | Image not found<br>http://www.worldagroforestrycentre.org |
| *Prunus spp* | 630 |  <br>http://visualsunlimited.photoshelter.com/image/I0000KjSKhXulitc<br>http://www.worldagroforestrycentre.org |

# ANNEX B:

Calculation of wood moisture content

To calculate the percentage of water in the wood (moisture content), it is necessary the initial mass of the test wood samples. Upon drying in an incubator at 80 ºC, the wood dry weight is calculated, according to the following formula:

mi = Initial mass

mf = Final mass

WC = water content

$$WC = \frac{mi - mf}{mi} \times 100$$

Some loss of mass can append in the dry, on that case this calc is used:

mi = initial mass

fdw = final mass of wet block after drying

WC = water content (%)

$$\frac{(mi - WC) - Fdw}{Mi - WC} \times 100$$

# ANNEX C:

Figures of isolated wood cells, after degradation by fungi.

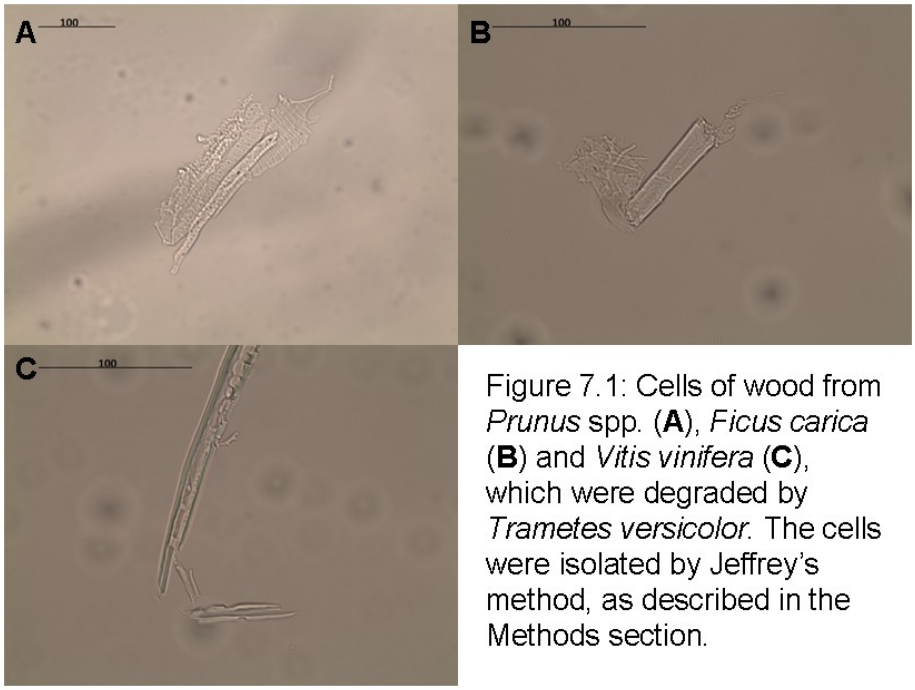



Figure 7.1: Cells of wood from *Prunus* spp. (**A**), *Ficus carica* (**B**) and *Vitis vinifera* (**C**), which were degraded by *Trametes versicolor*. The cells were isolated by Jeffrey's method, as described in the Methods section.

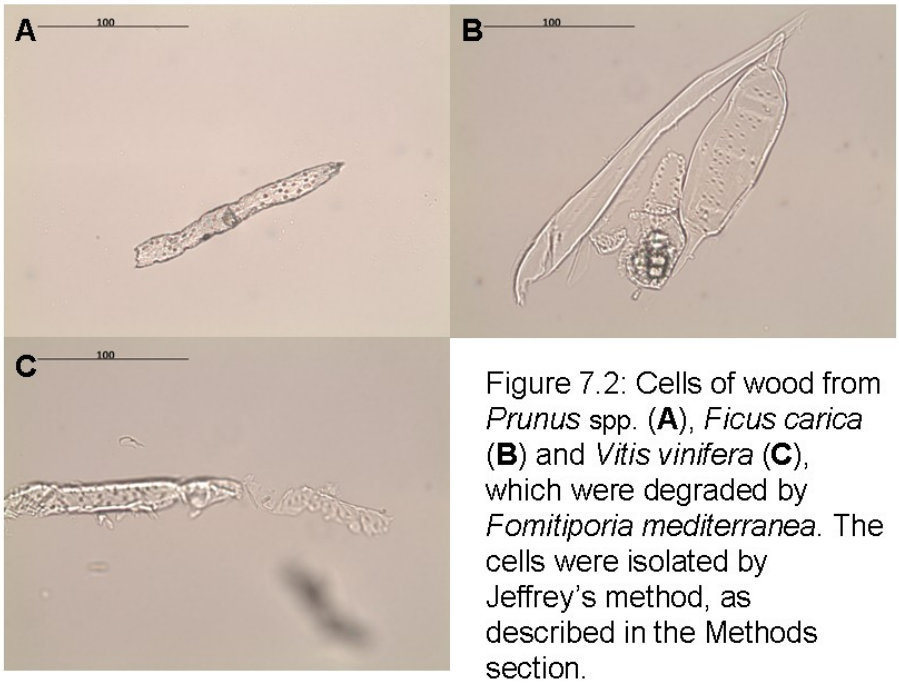



Figure 7.2: Cells of wood from *Prunus* spp. (**A**), *Ficus carica* (**B**) and *Vitis vinifera* (**C**), which were degraded by *Fomitiporia mediterranea*. The cells were isolated by Jeffrey's method, as described in the Methods section.

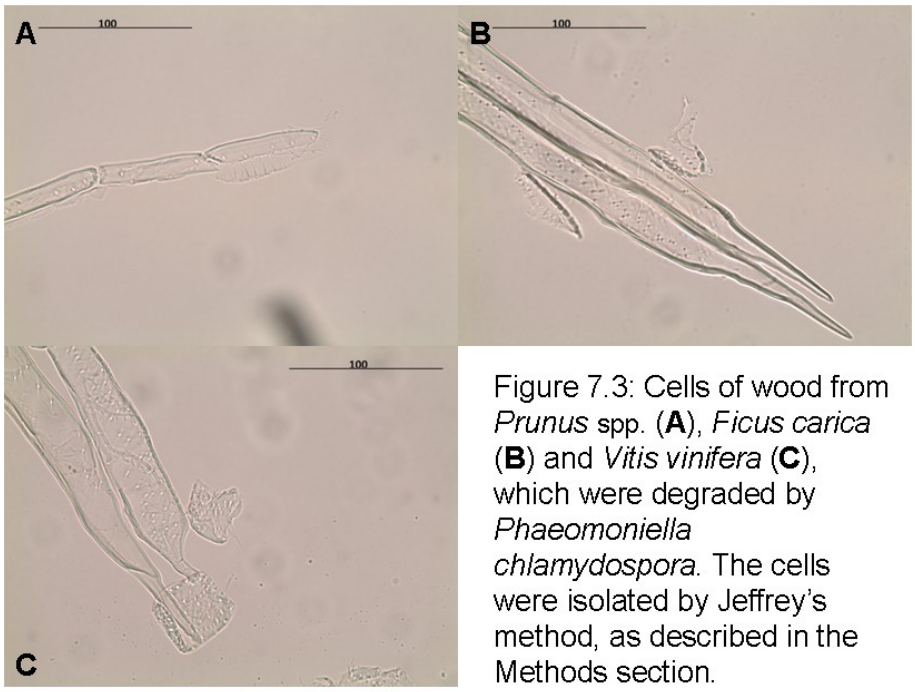



Figure 7.3: Cells of wood from *Prunus* spp. (**A**), *Ficus carica* (**B**) and *Vitis vinifera* (**C**), which were degraded by *Phaeomoniella chlamydospora*. The cells were isolated by Jeffrey's method, as described in the Methods section.